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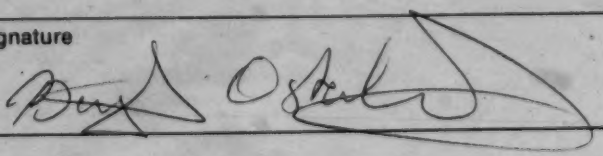

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A STABILIZING FACTOR FOR ATP CITRATE LYASE

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

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THE UNIVERSITY OF ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled A STABILIZING FACTOR FOR ATP CITRATE LYASE submitted by BENGT RUNE ÖSTERLUND in partial fulfilment of the requirements for the degree of

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ABSTRACT

ATP citrate lyase has been purified from rat liver to apparent homogeneity as judged from the appearance of one protein band on gel electrophoresis in the presence of sodium dodecyl sulfate. A stabilizing factor, previously reported to protect phosphofructokinase against thermal and lysosomal inactivation, has been isolated from rat liver and is shown to be equally effective on ATP citrate lyase. Nicking of ATP citrate lyase, caused by a lysosomal extract, is not prevented by the stabilizing factor, but further proteolytic degradation with concomitant loss of activity is retarded. The level of the stabilizing factor has been found to be inversely correlated with the rate of degradation of phosphofructokinase and it has been suggested that it is a physiological regulator of the degradation of phosphofructokinase, since neither glucokinase or pyruvate kinase is affected. Malic enzyme, purified to homogeneity from rat liver, is also unaffected by the stabilizing factor.

The stabilizing factor has been partially purified and characterized in order to determine its identity. The size of the factor, originally reported to be approximately 3,500, is less than 500 as judged by gel exclusion chromatography provided that glucose is not present in the elution buffer. Many of its properties are reminiscent of the tripeptide glutathione; the amino acid composition of the preparation consists of an equal molar ratio of glu, gly, half-cys; the N-terminal residue is found to be glu; the

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preparation is resistant to standard sequencing methods, suggesting a γ-linkage between glu and the penultimate residue. The stabilizing factor is, however, not identical to glutathione, since much higher concentrations of GSH are needed to protect ATP citrate lyase against thermal inactivation and furthermore no free sulfhydryl groups can be detected.

The thermal inactivation of ATP citrate lyase and the mode of action of the stabilizing factor in protecting this process have been investigated. It is suggested that the inactivation of ATP citrate lyase is coupled with oxidation of especially vulnerable sulfhydryl groups important for activity, since the process can be reversed by reducing reagents. The stabilizing factor prevents this oxidation but once the enzyme is oxidized the factor is without effect. The stabilizing factor is also able to protect the enzyme against inactivation by iodoacetamide.

The stabilizing factor is a very stable entity, resistant to boiling, to pronase digestion, and to treatment with hydroxylamine and iodoacetamide. In an attempt to identify the stabilizing factor, a number of compounds have been tested as stabilizers of the thermal inactivation of ATP citrate lyase. The substrates of the enzyme, citrate and CoA, can induce thermal stability to the enzyme. However, our factor solution has been found to be devoid of these compounds, by assaying for citrate and CoA, respectively, using procedures developed that are based upon the activity

of ATP citrate lyase. The specific identity of the factor therefore remains obscure.

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LIST OF ABBREVIATIONS

ADP	adenosine-5′-diphosphate
ATP	adenosine-5′-triphosphate
CoA	Coenzyme A
cyclic-AMP	adenosine 3′:5′-cyclic monophosphoric acid
db-cyclic-AMP	$N^6,O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphoric acid
DTNB	5,5-dithio-(bis)-nitrobenzoic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetate
GSH	reduced glutathione
GSSG	oxidized glutathione
GTF	glucose tolerance factor
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
PITC	phenylisothiocyanate
SDS	sodium dodecyl sulfate
TLC	thin layer chromatography
TLCK	tosyl-L-lysine chloromethyl ketone
TPCK	L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone
Tris	tris-(hydroxymethyl)aminomethane

I. INTRODUCTION

The flow of carbons from glucose into fatty acids and triglycerides is known as lipogenesis. Although lipogenesis is predominantely a cytosolic process, the mitochondria plays an important role in the mammalian species. Pyruvate, the endroduct of the glycolytic pathway, is converted to citrate in the mitochondria by the concerted action of pyruvate dehydrogenase and citrate synthetase. Citrate is transported to the cytosol and there it is cleaved by a specific enzyme, ATP citrate lyase, formerly called citrate cleavage enzyme, into acetyl-CoA and oxaloacetate (see Eqn. [1]).

$$\text{ATP} + \text{citrate} + \text{CoA} \rightleftharpoons \text{oxaloacetate} + \text{acetyl-CoA} + \text{ADP} + P_i \qquad [1]$$

Evidence has been presented which indicates that over 80% of the extramitochondrial acetyl-CoA formed from pyruvate via the mitochondria is supplied through the ATP citrate lyase pathway (1,2). Acetyl-CoA so formed can now be used for the production of fatty acids, by the two cytosolic enzymes, acetyl-CoA carboxylase and the fatty acid synthetase complex.

The switch of the mammalian liver from an organ for fatty acid synthesis to an organ for fatty acid oxidation is under both hormonal and dietary control, responding acutely to both the energy and the fatty acid requirements of the body as a whole. This is also reflected in the capacity of

fatty acid synthesis which is known to vary within wide ranges in response to nutritional and hormonal extremes. Thus, starvation decreases the rate of fatty acid synthesis to very low levels, and refeeding starved animals with a diet high in carbohydrate and low in fat induces the rate of fatty acid synthesis to levels that are one order of magnitude above normal levels (3-7). The lipogenic capability of a diabetic animal is similarly depressed and this is restored by insulin therapy; when insulin is given in high doses the rate of fatty acid synthesis rises to supranormal levels. The activity of several lipogenic enzymes are known to decrease or increase in parallel with the rate of lipogenesis (6-11). Among these are glucokinase phosphofructokinase, pyruvate kinase, ATP citrate lyase, acetyl-CoA carboxylase, and fatty acid synthetase. Three other lipogenic enzymes are involved in the production of reducing equivalents needed for fatty acid synthesis. These are malic enzyme, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

In the lipogenic pathway one encounters a multitude of control mechanisms, regulating the overall rate of production of fatty acids. These mechanisms can be divided into two types: 1) short-term control, related to changes in substrate, cofactor and product concentrations or inactivation of existing enzyme molecules, and 2) long-term control involving alteration of enzyme quantity (8). Sometimes it is difficult to distinguish between these two

types. For instance, in the dietary manipulation of an animal such as refeeding starved rats with a high carbohydrate-low fat diet, one can easily envision both types of regulatory mechanisms operating simultaneously even though long-term control seems to override the short-term. It is entirely possible that the control mechanisms operate sequentially, such that short-term control initially gears the pathway to an increase in carbonflux and that long-term control takes over adapting the pathway to a constant high load of carbohydrates.

Enzyme activity can be modulated by changes in the concentration of substrates, cofactors and products, as well as by allosteric effectors. Acetyl-CoA carboxylase is considered to be the rate-limiting enzyme in the short-term control of fatty acid synthesis (12). This is substantiated by the finding that citrate and long-chain acyl-CoA act as allosteric regulators of this enzyme. Citrate activates acetyl-CoA carboxylase and this activation is accompanied by aggregation of inactive protomers to active polymeric filaments (13). Long-chain acyl-CoA in micromolar concentrations inhibits the enzyme competitively with citrate (13). Also long-chain acyl-CoA inhibits specifically the mitochondrial citrate transport system (14) thereby reducing the amount of citrate available for activation of acetyl-CoA carboxylase. Changes in the concentration of citrate and long-chain acyl-CoA thioesters in various physiological conditions are generally consistent with the proposed

regulatory roles of these allosteric effectors (15-17). The significance of citrate activation is controversial, however, since the cytoplasmic concentration of citrate seems to be too low (18) and some investigators failed to detect changes in citrate concentration during induction of lipogenesis (17,19).

ATP citrate lyase, the enzyme which precedes the acetyl-CoA carboxylase and uses citrate as a substrate to form acetyl-CoA is inhibited by ADP, a product of the reaction. This inhibition is competitive with ATP (20) and this has led Atkinson (21) to propose that ATP citrate lyase is under the control of the energy charge of the cell. However. since the energy charge is very constant during dietary manipulation (12,17) the control of ATP citrate lyase by this mechanism is unlikely.

Firm evidence for metabolite control of fatty acid synthetase is scarce. The proposed inhibitory effect of palmitoyl-CoA (22) was later shown to be a detergent effect (23). Fructose-1,6-diphosphate has been found to be an activator of fatty acid synthetase (24), but at concentrations far above those found in the tissue.

Another set of control mechanisms that could be involved in the early onset of adaptive changes initiated by dietary or hormonal manipulation is the activation of dormant enzymes. It has been shown (25,26) that in rat liver during the first three hours of refeeding after a 48 hour fast, an enzymatically inactive, immunologically reactive

fatty acid synthetase is present. The conversion of this apo-enzyme to holo-enzyme by incorporation of 4'-phosphopantetheine has been demonstrated both in liver extracts from rat (26) and by incubation of purified apo-enzyme from pigeon liver with ATP, CoA and a pigeon liver enzyme system (27). Possible physiological significance of this process as a regulatory mechanism is substantiated by rapid turnover of 4'-phosphopantetheine which decreases in liver during starvation (28). A similar system of apo- to holo-enzyme conversion has been suggested for regulation of acetyl-CoA carboxylase activity, where the incorporation of the biotinyl prosthetic group, precedes the polymerization of the enzyme to the active form (29). The importance of biotin has been demonstrated in the adipose conversion of 3T3 cells in medium supplemented with serum. The morphological changes of these cells are accompanied by the accumulation of triglycerides, a process requiring the presence of biotin in the serum (30).

The induction of lipogenesis that occurs by dietary manipulation could conceivably be attributable to the increase in circulating glucose, which might exert a direct stimulatory role on lipogenesis (31). On the other hand, it is known that glucose stimulates insulin release before any changes can be detected in the intracellular steady-state levels of glucose metabolites (32). Furthermore, a wealth of evidence indicates that insulin is directly involved in the control of lipogenesis, both in short-term and long-term

control. The antagonistic actions of insulin and glucagon are well documented. While the intracellular response to glucagon is mediated by the "second messenger", cyclic-AMP, (33), we are ignorant of any intracellular mediator of the insulin response (34). Insulin-receptor association produces an increase in glucose transport capability of the cell membrane, but it is not clear how this process could account for all of the insulin-mediated cellular responses. The hypothesis that cyclic-AMP may mediate insulin action has recieved strong support from the demonstration that insulin counteracts the glucagon induced production of cyclic-AMP in the perfused liver (33). Moreover, insulin can actually lower the concentration of cyclic-AMP if it is already high (35). The observation that insulin may cause a decrease in the level of cyclic-AMP, while at the same time cause a marked but transient rise in cyclic-GMP levels in fat cells, has led to the "unitary cyclase" hypothesis, whereby a single nucleotide cyclase enzyme catalyzes the formation of cyclic-AMP or cyclic-GMP depending on the nature of the hormone antagonist (36). That cyclic-GMP is involved in the action of insulin is not generally accepted, however, since agents that increase cyclic-GMP levels fail to elicit any of the insulin sensitive parameters in fat cells and liver cells (34).

A role for cyclic-AMP both in the short-term and the long-term regulation of lipogenesis has been suggested. Glucagon and db-cyclic-AMP give rise to a rapid decline in

the rate of fatty acid synthesis, which is matched by a similar decline in the activity of acetyl-CoA carboxylase (37-39). The work by Kim and coworkers (40,41) suggests that this inactivation is due to a phosphorylation of the enzyme by a protein kinase. Rapid modification of enzyme activity by phosphorylation-dephosphorylation reactions was first known for enzymes in the glycogen metabolism (33) and the number of enzymes known to be modified this way is increasing all the time (42). Of the lipogenic enzymes, in addition to acetyl-CoA carboxylase, phosphofructokinase (43) and pyruvate kinase (44) have been reported to undergo phosphorylation-dephosphorylation reactions. Phosphorylation of fatty acid synthetase has been demonstrated in pigeon liver (45), but attempts to verify this modification in the liver of rats or humans were unsuccessful (46,47). Another site for cyclic-AMP action is triglyceride lipase, which is activated by phosphorylation catalyzed by a cyclic-AMP dependent protein kinase (48). This activation would lead to increased levels of long-chain acyl-CoA in the cell, possibly accounting for the observed inhibition of lipogenesis by glucagon.

Measurements have been made of hormone levels in the blood and concentration of intracellular metabolites including cyclic-AMP in rat liver during starvation and early refeeding (17). The results suggested that the ratio of insulin to glucagon was responsible for the regulation of glucose metabolism via control of cyclic-AMP and long-chain

acyl-CoA concentrations.

Long-term adaptation introduces yet another control of lipogenesis, namely regulation of the number of active enzyme molecules, and thereby changing the flow of metabolites through the pathway. By combination of specific immunoprecipitation and radioactive labelling techniques it has been shown that the long-term fluctuation of the activity of the lipogenic enzymes during dietary and hormonal manipulation is due to a change in the content of enzyme proteins (10,49-58). The induction has been shown to be associated with an active net uptake of radioactive isotope into the lipogenic enzymes. It is noteworthy that these changes in concentration of specific enzymes take place against a continual background of protein turnover, which may also be subject to alteration by nutritional and hormonal stimuli.

That proteins are biologically dynamic compounds was first demonstrated in 1942 (59). Both the rate of synthesis and the rate of degradation governs the tissue concentration of a protein; an alteration in either of these rates will change the level of the enzyme. A simple model that shows this relation has been developed (60). While enzyme synthesis follows zero-order kinetics, it has been found consistently that degradation shows first-order kinetics. Thus a change in enzyme content may be expressed as:

$$\frac{dE}{dt} = k_s - k_d E \qquad [2]$$

where E is the content of enzyme per unit weight, k_s the rate constant for synthesis, expressed as enzyme units/time and k_d the first-order rate constant for enzyme degradation, expressed as time^{-1}. When the enzyme exists in a steady-state there is no change of enzyme with time, i.e. $dE/dt = 0$ and Eqn. [2] reduces to:

$$E = \frac{k_s}{k_d}$$

Thus under steady-state conditions the level of E will be determined by the relative rates of synthesis and degradation. If now the enzyme level is changed to a new steady-state level by some physiological stimulus, k_s and k_d will then change to new values k_s' and k_d' and the ratio k_s'/k_d' will now determine the new steady-state level E'. The time course of approach to E' can easily be worked out, if we assume that the rate constants change instantaneously to the new values k_s' and k_d'. Eqn. [2] then becomes:

$$\frac{E'}{E} = \frac{k_s'}{k_d'E} - \left(\frac{k_s'}{k_d'E} - 1\right) e^{-k_d't} \qquad [3]$$

From [3] it can be seen that the time required to approach a new level of enzyme is a function only of the rate constant for degradation (60). The time taken to obtain one-half of the final level is defined as the half-life, t1/2, of the enzyme. An expression for the half-life can easily be obtained from Eqn. [3] by setting the initial enzyme level E equal to 1 and knowing that the final level E' is equal to k_s'/k_d'. One-half of the total change is then equal to $1/2(k_s'/k_d' - 1)$ and Eqn [3] becomes:

$$\frac{1}{2}\left(\frac{k_s'}{k_d'} - 1\right) = \frac{k_s'}{k_d'} \quad \left(\frac{k_s'}{k_d'} - 1\right) e^{-k_d't}$$

which reduces to:

$$-\frac{1}{2} = e^{-k_d't}$$

and solving for t gives:

$$t = \frac{\ln 2}{k_d'}$$

It is possible then, to estimate experimentally the half-life of any enzyme by following its time course of change to a new steady-state level. Such a kinetic analysis has been performed with a variety of adaptable enzymes (9,61).

An adaptive response involving a change in the amount of the enzyme could theoretically be regulated by alteration either of the rate of synthesis or the rate of degradation, or both. The kinetic method discussed above, together with newer techniques of radioisotope incorporation with immunological procedures to isolate the specific enzyme, have pointed to a predominant role of accelerated rate of enzyme synthesis in establishing high levels of the lipogenic enzymes upon refeeding starved animals. Furthermore, a decreased rate of synthesis as well as an increase in the rate of degradation brings about the low levels of enzyme during starvation (10,50,53,62)

While the method of kinetic analysis carries certain assumptions and limitations, the radioisotope techniques invariably suffer from the drawback of isotope reincorporation leading to an underestimation of the degradation rate

(61,63). The experimental time periods involved in these measurements are fairly long especially for proteins with longer half-lives, and thus variations in the rate of degradation could go undetected. It has recently become clear that the apparent rate constants k_s and k_d are functions of time during nutritional stimulus (64). The way in which k_s and k_d vary with time would then determine the enzyme accumulation profile, in such a way that an increase in k_s together with a transitory decrease in k_d would lead to a more rapid attainment of the final enzyme level (65). Such coordination of k_s and k_d would give the system enhanced flexibility (64,65). The absolute values of the rate constants are also important, since an enzyme with a short half-life would adapt faster than an enzyme with a relative longer half-life. Enzymes located at important control points where they regulate the flow of substrates through a metabolic pathway, might then evolve with especially short half-lives (61). Comparisons of measured half-lives of various enzymes confirms this concept (61).

The molecular events underlying the regulation of enzyme levels through alteration in the rate of synthesis and/or the rate of degradation are not well understood. The details of protein biosynthesis are now well delineated and several sites for metabolic control have presented themselves (67). The route(s) for the breakdown of proteins, however, is still elusive and so is the regulation of this process. The fact that degradation of a specific enzyme *in*

vivo follows first-order kinetics implies that the protein molecules are removed or degraded in a random fashion (68), which suggests that once a protein molecule is synthesized, the product can immediately be a substrate for degradation. The variability of degradation rates among different proteins (66) within a cell further suggests that the property of the protein being a substrate for a degradative process is inherent in the three-dimensional structure of the protein.

Several investigators have tried to correlate degradation rate constants with various physicochemical properties of proteins. Thus half-lives of proteins have been found to correlate with size (69) and charge (70) and with a parameter calculated from both size and amino acid composition (71). It has been suggested that the rate limiting step in protein degradation is an initial denaturation (72). In support of this conclusion is the finding that proteins with short half-lives tend to be inactivated easier by temperature (73) and by acid pH (74). These parameters might reflect a more fundamental aspect of protein structure, such as compactness of folding, which may determine the half-life, since unfolding of a protein tends to increase the susceptibility of the protein to protease attack (91). *In vitro* experiments have shown that shortlived proteins are more susceptible to proteolytic attack by various well-characterized proteases (75) or lysosomal proteases (76,77).

Despite the above correlations, many exceptions have been found and it is possible that a very slight modification to a protein can have a large effect on its degradation rate. This is particularly evident in studies where amino acid analogues are incorporated into proteins. The resulting proteins are normally degraded *in vivo* much faster than the native protein (66).

The elucidation of the regulation of protein degradation will first require a description of the steps which lead from active protein to its constituent amino acids. Of particular interest are the identification of the rate limiting steps and the nature of factors that may regulate them. It has been proposed that a protein can exist in different conformations which may be reversibly or irreversibly connected, and that only one or some of these forms are susceptible to degradation (61,78,79). If the rate limiting step were the conversion of the protein to a more labile form, this step could be a control point in protein degradation. The equilibrium between conformers could be altered by interactions with ligands. There are numerous examples of such ligands either stabilizing or labilizing specific enzymes (66). For instance, tryptophan oxygenase was found to be induced by the administration of its substrate tryptophan, which also stabilized and prevented the degradation of the enzyme in the presence of continued synthesis (67). Further, the degradation of pyridoxal phosphate containing enzymes may be limited by the rate of

cofactor dissociation from the holo-enzyme (81), and the rate of inactivation of acetyl-CoA carboxylase by a lysosomal extract is decreased by citrate and increased by palmitoyl-CoA (82). A factor was recently found in rat liver which stabilized phosphofructokinase from inactivation by temperature and by lysosomal extract (83). The level of this factor was found to correlate with the dietary and hormonal status of the animal. For example, the low levels found in fasting and in diabetic animals increased dramatically in response to feeding (83) and insulin therapy (128), respectively, to far above the normal state. The fact that the degradation rate of phosphofructokinase is inversely related to the tissue level of the factor (50,128), is in keeping with the concept that the factor may play an important physiological role in modulating the half-life of phosphofructokinase.

The lysosome has been widely assumed to be the site of protein degradation because of its high content of proteolytic activity (78). The accessibility of the substrates to the hydrolytic enzymes in the lysosomes has been explained by the process of engulfing of intracellular matter (not only soluble macromolecules, but also organelles and membraneous structure) within a membrane to form what is called an autophagic vacuole. These vacuoles have the ability to fuse with lysosomes to form secondary lysosomes (or phagosomes). This system would provide the cell with the capacity for bulk removal of proteins and could play a major

role in maintaining the availability of amino acids for purposes such as gluconeogenesis during starvation. Consistent with this idea is the finding that the production of glucose from amino acids, promotes the formation of autophagic vacuoles (97). If protein degradation were confined to the lysosome route, with prior vacuolization, either the degradation *per se* or the vacuolization would have to be able to discriminate between different proteins. On the other hand, if protein degradation occurs extralysosomally at least in part, the wide range of half-lives of proteins could more easily be rationalized (78). The discovery of cytoplasmic group-specific proteases specific for the apo-form of pyridoxal enzymes (79) supports this notion.

Regulation of ATP citrate lyase

Of the enzymes involved in *de novo* fatty acid synthesis, acetyl-CoA carboxylase has been studied most extensively. It is generally considered to be the rate limiting enzyme, subject to precise control by covalent modification and allosteric effectors thereby regulating the rate of production of malonyl-CoA for fatty acid synthesis. However, an exclusive regulatory role has been questioned (12). The starting precursor for fatty acid synthesis and also cholesterol synthesis is acetyl-CoA which is generated in the cytosol by the activity of ATP citrate lyase. Like

other lipogenic enzymes the activity of this enzyme has been shown to be markedly stimulated by refeeding starved rats or by insulin treatment of diabetic rats. The adaptive behaviour of ATP citrate lyase has been considered paradoxical since in the early stages of starvation and refeeding there is no correlation between activity and the rate of fatty acid synthesis (84). This would indicate that ATP citrate lyase is not regulating the rate of fatty acid synthesis, as has been suggested by Spencer et al (85), but rather that the rate of fatty acid synthesis may control the amount of ATP citrate lyase (84).However it has recently been shown by Rognstad (98) that the hepatic activity of ATP citrate lyase in resting rats, while not quite limiting, is insufficient to support the burst of lipogenesis that follows refeeding. In other words, if it were not for the burst of ATP citrate lyase, activity of this enzyme would clearly influence the lipogenic capacity.

It is the purpose of this study to further explore the role of ATP citrate lyase in lipogenesis and specifically to investigate what factors if any, are responsible for the increase in activity during dietary and hormonal manipulation.

Studies concerning the role of cyclic-AMP in influencing the induction of ATP citrate lyase activity are described in chapter III. The purification of ATP citrate lyase, and also the malic enzyme are reported in chapter IV. During early attempts to purify ATP citrate lyase, we were

annoyed by the instability of the enzyme and therefore decided to test if the stabilizing factor for phosphofructokinase described by Dunaway and Segal (83) had any effect on ATP citrate lyase. We found that the factor had a dramatic stabilizing effect on ATP citrate lyase against thermal and lysosomal inactivation. These experiments are discussed in chapter V. Tests on the general stability characteristics of malic enzyme and the effect of the stabilizing factor are also reported in this chapter. In order to understand the physiological role of the stabilizing factor and the nature of its interaction with ATP citrate lyase, the stabilizing factor was isolated from rat liver and further purified (chapter VI) and finally attempts were made to characterize the factor as to its chemical identity and mode of action (chapter VII).

II. GENERAL MATERIALS AND METHODS

A. MATERIALS

1. Animals.

Male white Woodlyn/Wistar rats or Sprague-Dawley rats were used. No differences has been noted between the strains. The rats were housed in steel cages, had access to fresh water at all times and were fed *ad libitum* with a pelleted balanced diet (Purina Lab Chow) for at least 7 days prior to use.

2. High carbohydrate diets.

High carbohydrate, low-fat diet was supplied in a pelleted form by United States Biochemical Corporation, Cleveland, Ohio, U.S.A. under the name "High Carbohydrate Test Diet" and contained 68% sucrose, 8% fat (cotton seed oil), 18% casein, 4% salt mixture and 2% vitamin mixture and brewers yeast.

High carbohydrate, fat-free diet was prepared in a pelleted form to our specification by the same company. This diet contains 63% sucrose, 24% casein, 2.5% cellulose powder, 2.5% dried brewers yeast, 1% glycine, 0.5% DL-methionine, 0.025% antioxidant, 0.3% chromic acid, 0.6% vitamin mixture and 5% salt mixture.

3. Chemicals.

Sephadex G-15, G-25, G-50, G-200, DEAE-Sephacel and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals, Sweden. DE52-cellulose was purchased from Whatman Ltd., England and Biogel P2 from BioRad Lab., U.S.A. Polyethylene Glycol 6,000 and hydroxylamine hydrochloride were supplied by Baker Chemical Co., U.S.A. Ultrapure ammonium sulfate was obtained from Schwarz/Mann, U.S.A., Zn-insulin from Connaught Lab. Ltd., Canada and TPCK-treated trypsin from Worthington Biochemical Corp., U.S.A.

Db-cyclic-AMP, cyclic-AMP, aminophylline, theophylline, 3-isobutyl-1-methylxanthine, iodoacetamide, DTNB, fluorescamine, TLCK, chymotrypsin, CMC-pronase, malate dehydrogenase (pigeon breast muscle), and protamine sulfate were all purchased from Sigma Chemical Co., U.S.A. Alloxan was a gift from Dr. N. Madsen of the University of Alberta.

B. METHODS

1. Enzyme assays.

a. ATP citrate lyase.

Enzyme activity in the direction of citrate cleavage, was assayed by coupling to the malate dehydrogenase reaction, as described by Inoue *et al.* (20). The assay mixture contained 200 mM Tris-HCl, pH 8.4; 20 mM potassium citrate; 10 mM $MgCl_2$; 5 mM DTT; 2 units of malate dehydrogenase per ml; 0.14 mM NADH; 5.25 mM ATP; in a total volume of 1.02 ml. After the addition of enzyme (5-25 µl) the

reaction was initiated with 10 µl of 10 mM CoA and the rate of NADH oxidation was measured at 340 nm on a Cary 15 spectrophotometer. All assays were carried out at 25°. One unit of enzyme is defined as the amount of enzyme, that catalyzes the oxidation of 1 µmole of NADH / minute under these conditions. All activity measurements were performed on the initial linear decrease in NADH oxidation.

b. Malic enzyme.

Enzyme activity was measured in the direction of malate oxidation as described by Wise and Ball (119). The standard assay mixture contained 70 mM Tris-HCl, pH 7.4; 5 mM L-malate (neutralized with NaOH) 1 mM $MgCl_2$; and 0.1 mM NADP; in a total volume of 1.0 ml. 5-15 µl enzyme was added to initiate the reaction and the rate of NADPH formation was measured in a Cary 15 spectrophotometer. All assays were carried out at 25°. One unit of enzyme is defined as the amount of enzyme, that catalyzes the formation of 1 µmole of NADPH / minute under these conditions.

2. Determination of protein concentration.

Protein concentrations, during the enzyme purification and of the lysosomal extract, were determined by the method of Lowry *et al* (127), using bovine serum albumin as standard. For other studies which made use of the pure

enzymes, the extinction coefficient $E^{1\%}_{1\,cm}$ at 279 nm of 11.4 for ATP citrate lyase (as determined by Singh *et al*) (123). and of 8.6 for malic enzyme (as determined by Hsu and Lardy for pigeon liver malic enzyme) (121) were used. The validity of these values were tested before use.

3. Amino acid analysis.

The stabilizing factor was dialyzed against water for 24 hours and then lyophilized. Acid hydrolysis, with and without prior performic acid oxidation, was performed in 6 N HCl, containing 0.1% phenol in evacuated tubes at 110° for 22 hours. Any remaining carbohydrate, which resinified, was removed by centrifugation and the resultant supernatant was taken to dryness and subjected to analysis on a D500 Durrum Amino Acid Analyzer (176). The N-terminal amino acid was dansylated and identified according to Hartley (177).

4. SDS gel electrophoresis.

The procedure of Weber and Osborn (136) was used for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, using gels with half the normal amount of crosslinker.

III. REGULATION OF ATP CITRATE LYASE BY CYCLIC-AMP

A. INTRODUCTION

A starvation-refeeding schedule will stimulate metabolic control mechanisms in such a way that homeostasis is conserved. Thus during starvation, blood glucose levels are maintained by release of glucose from the liver, while energy is obtained mainly from oxidation of fatty acids (88). During starvation, the glucagon/insulin ratio in the blood is elevated and this is correlated with high levels of intracellular cyclic-AMP (86). This mediator rapidly stimulates glycogen breakdown in the liver (87) and mobilization of fatty acids from adipose tissue (48) by way of activating glycogen phosphorylase and triglyceride lipase, respectively. It is also believed that this nucleotide is responsible for the increase in the rate of hepatic gluconeogenesis by stimulating the synthesis of phosphoenolpyruvate carboxykinase to levels above normal (35,89,90).

All the effects caused by starvation are reversed by refeeding (17,92). Furthermore, if the diet is rich in carbohydrate, the rates of triglyceride formation and lipogenesis are increased to levels far above normal. This increase is closely paralleled by an increase in the activity of the lipogenic enzymes. Several lines of evidence suggest that cyclic-AMP plays an important role during this induction. Administration of glucagon or db-cyclic-AMP

during refeeding will prevent the decrease in cyclic-AMP level. The rapid decrease in phosphoenolpyruvate carboxykinase level is prevented (92) and the induction of several lipogenic enzymes is markedly decreased by such treatment (93-96), as is the rate of fatty acid synthesis (93).

More rapid effects of glucagon (37) and db-cyclic-AMP (38,39) can be seen in liver slices, where the inhibition of fatty acid synthesis is accompanied by a parallel decrease in the activity of acetyl-CoA carboxylase. This rapid effect could be attributable to a phosphorylation mechanism as suggested by Kim *et al* (40,41).

Because of its prime metabolic location connecting the glycolytic pathway with *de novo* fatty acid synthesis, ATP citrate lyase has the potential of regulating the flow of carbons in lipogenesis and therefore might be expected to be subject to precise control. The experiments described in this chapter were designed to determine if cyclic-AMP is involved in the control of the induction of ATP citrate lyase during refeeding a high carbohydrate diet to starved rats. In view of the role of cyclic-AMP in modulating enzyme activity by covalent modification, *in vitro* experiments with ATP citrate lyase were performed to test if this type of control mechanism could be detected.

B. METHODS

1. Animals.

Male Woodlyn/Wistar rats (Woodlyn Lab., Guelph, Ontario) weighing about 340 - 390 gram, were used for the experiments described in this chapter.

2. Refeeding experiments.

Rats were starved for 48 hours and then refed either a high carbohydrate, low-fat diet or a high carbohydrate, fat-free diet.

Starvation and refeeding was started at 8 a.m. and the intraperitoneal administration of phosphodiesterase inhibitors was performed three times daily, at 8 a.m., 12 noon and 4 p.m. Db-cyclic-AMP, 4 mg and theophylline, 20 mg were dissolved in 1 ml 0.9% NaCl solution. Isobutylmethyl-xanthine was dissolved in 0.9% NaCl to a final concentration of 6.75 mM. The dosages of both solutions were 0.25 ml per 100 gram body weight each time.

After refeeding schedules of various durations, 1 - 3 rats were sacrificed by decapitation in the morning. Crude extract of rat liver was prepared as described by Inoue *et al* (20).

3. In vitro experiment.

Rats were starved for 24 hours and then made diabetic

by a single subcutaneous injection of a freshly prepared 3% aqueous solution of alloxan at a dosage of 15 mg per 100 gram body weight. After seven days animals with a blood glucose concentration of 500 mg per 100 ml or more (testing of the urine with Clinistix Reagent Strips, Ames Company, Rexdale, Ontario) were considered diabetic. Diabetic rats were given 10 units of Zn-insulin per day per rat intraperitoneally for two days. This treatment was found to give high levels of hepatic ATP citrate lyase activity.

Crude liver extract, prepared as above, was dialyzed overnight against 30 volumes of 10 mM Tris-HCl buffer (pH 7.8), containing 10 mM 2-mercaptoethanol and 1 mM $MgCl_2$.

Ammonium sulfate fractionated extract was prepared as described (20) and dialyzed as above. The protein fraction precipitating between 65 and 75% ammonium sulfate was also recovered (40) and dialyzed as above. This fraction contained no measurable ATP citrate lyase activity.

Crude extract (15 mg protein) or ammonium sulfate fractionated enzyme (12 mg protein) was incubated with or without the fraction precipitated between 65-75% ammonium sulfate (8 mg protein) at 30° in 10 mM Tris-HCl buffer pH 7.8, containing 10 mM 2-mercaptoethanol and 1 mM $MgCl_2$. The following were added either individually or in combination to final concentration as indicated: Aminophylline (1 mM), Cyclic-AMP (0.1 mM), ATP (1.8 mM) and $MgCl_2$ (6 mM). The activity of ATP citrate lyase was followed during a 2 hour incubation period.

C. RESULTS

1. Refeeding experiments.

Twice daily injections of a mixture of theophylline and db-cyclic-AMP during a refeeding schedule of starved rats with a high carbohydrate, low-fat diet partially prevents the induction of ATP citrate lyase (Fig.1). The maximum level of activity is substantially lower than that obtained in the control rats. If the treatment was interrupted after three days the activity of ATP citrate lyase was found to increase to high levels, suggesting that the removal of the inhibitors allowed cyclic-AMP to return to low levels (99), thereby removing the inhibitory effect on the induction of ATP citrate lyase. The drug isobutylmethylxanthine (100) was also found to prevent the induction of ATP citrate lyase (filled circles in Fig. 1).

The extent of the induction of ATP citrate lyase depends on the composition of the diet. As can be seen from Fig. 2, ATP citrate lyase activity increases to much higher levels if the high carbohydrate, low-fat diet is exchanged with a diet totally devoid of fat. This result confirms the earlier observation that fat in the diet leads to a dampening effect of the induction of lipogenesis and the lipogenic enzymes (101).

The injection of drugs into animals can cause stress and other effects which can become manifest in different ways. One such way is to affect appetite. A lower food intake could by itself produce a smaller induction of ATP

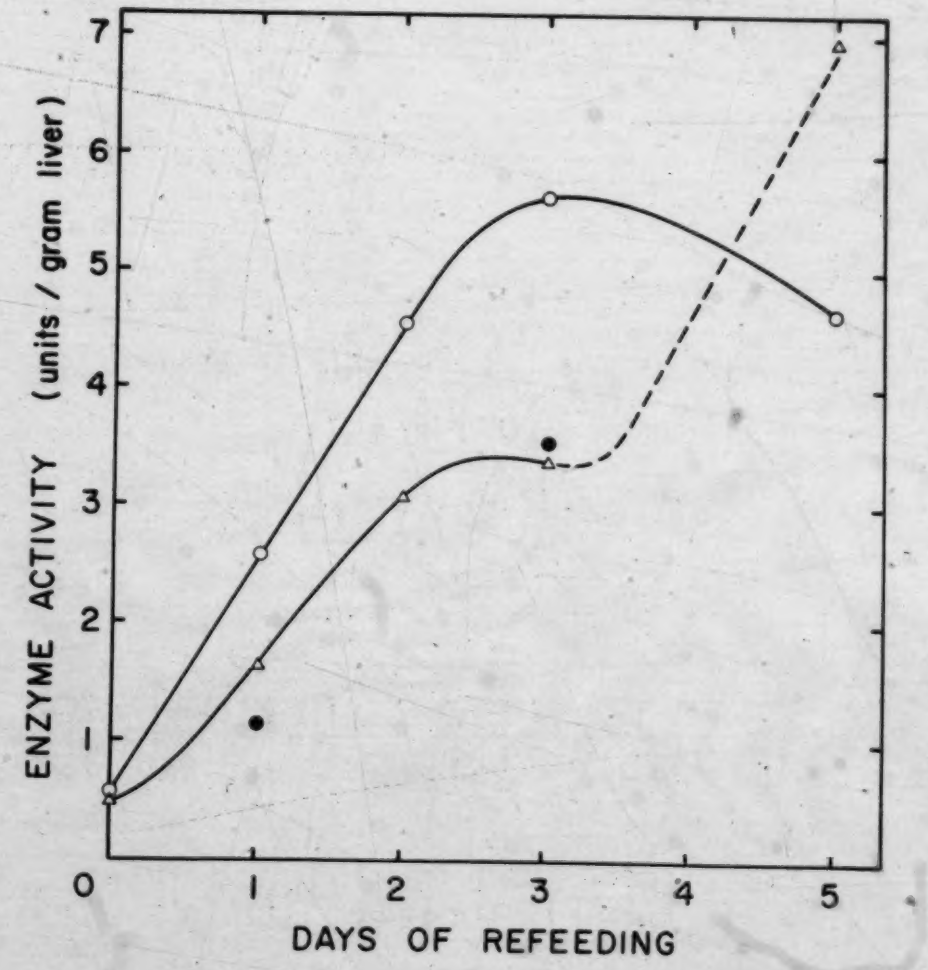

Fig.1. Effect of phosphodiesterase inhibitors on the induction of ATP citrate lyase caused by refeeding starved rats with a high-carbohydrate, low-fat diet.

Controls (○), treatment with a mixture of db-cyclic-AMP and theophylline (△), treatment with isobutylmethylxanthine (●).

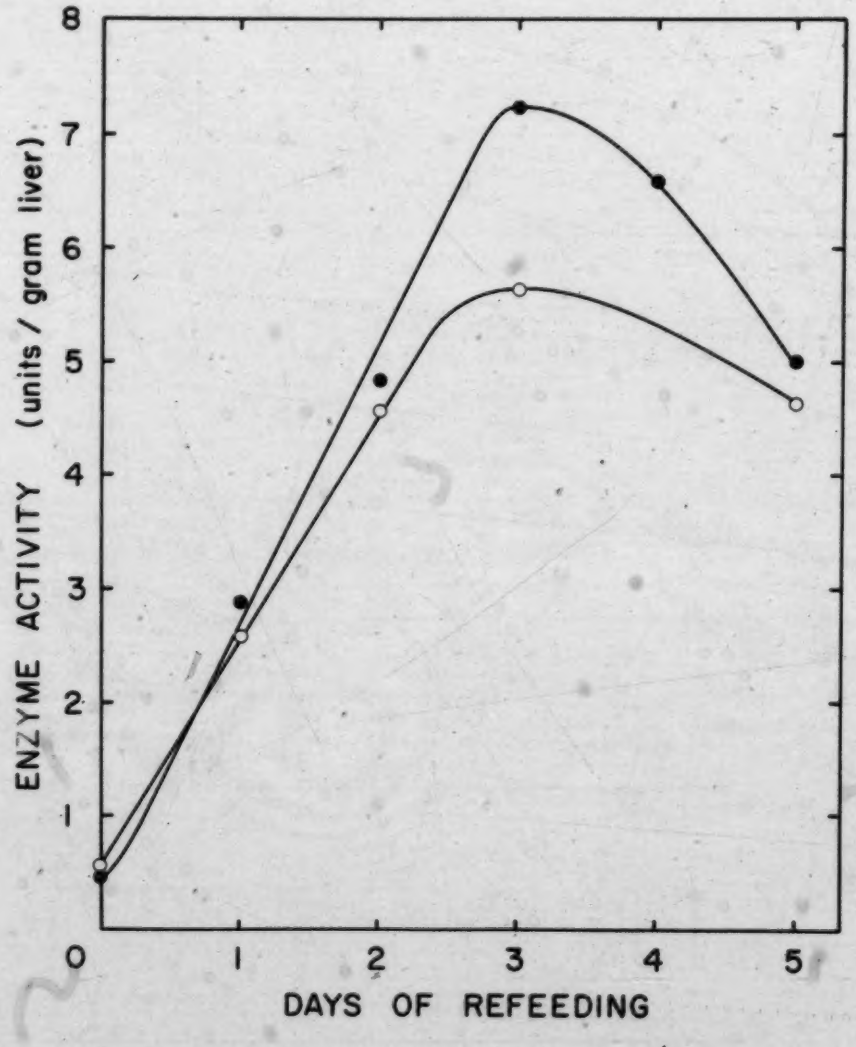

Fig.2. Effect of fat in the diet upon the induction of ATP citrate lyase by refeeding starved rats with a high-carbohydrate diet. High-carbohydrate diet containing 8% fat replotted from fig.1 (○), high-carbohydrate, fat-free diet (●).

citrate lyase. The experiments were therefore repeated and this time the food consumption was also measured. In the histogram (Fig. 3) it can be seen that rats injected with the phosphodiesterase inhibitors (right column) consume less than control rats (middle column). However the appetite returns when the administration of drugs is interrupted. The histogram also shows the food consumption of rats receiving a dietary regimen of a high carbohydrate, fat-free diet (left column) and the increase in consumption over the fat-containing diet is evident.

2. In vitro experiment.

If ATP citrate lyase is regulated by a phosphorylation-dephosphorylation mechanism, one would anticipate that modification would change some functional property of the enzyme. This modification could manifest itself by a change in the catalytic activity of the enzyme, as has been reported for other enzymes in carbohydrate and lipid metabolism.

Crude rat liver extract was incubated *in vitro* with or without cyclic-AMP and ATP-Mg, in the presence of aminophylline, a known inhibitor of cyclic-AMP phosphodiesterase. While the extract alone or in the presence of aminophylline showed a steady but slow decrease in the activity of ATP citrate lyase (90% remaining after 2 hours) neither cyclic-AMP or ATP-Mg, alone or in combination, affected the

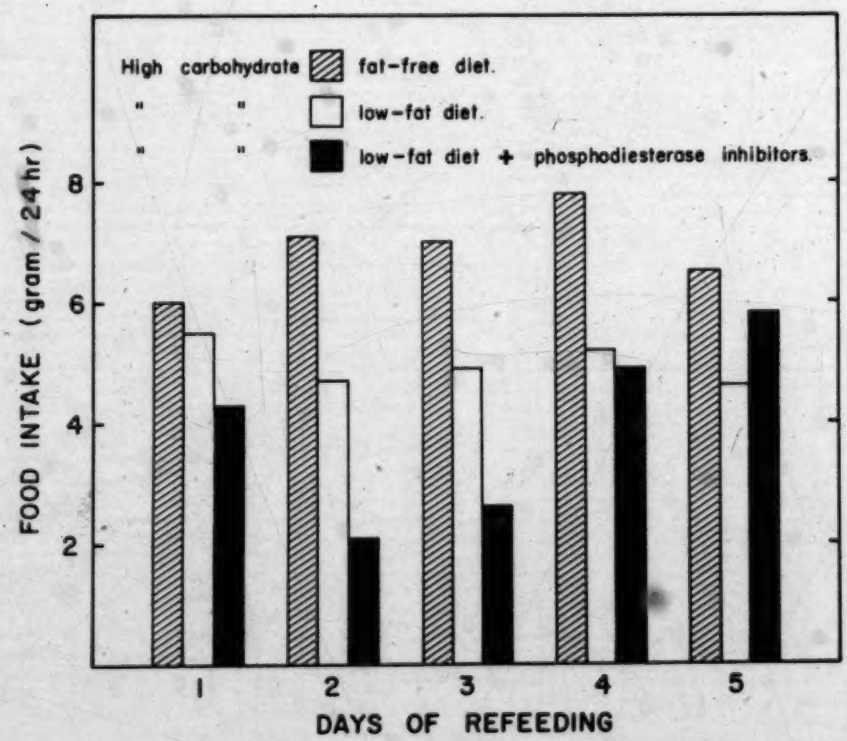

Fig.3. The daily food consumption during refeeding of starved rats with a high-carbohydrate, fat-free diet (left column), with a high-carbohydrate, low-fat diet (middle column) and with a high-carbohydrate, low-fat diet plus treatment with phosphodiesterase inhibitor during the first 3 days (right column).

enzymatic activity.

In testing a crude system, one can not exclude the possibility of the presence of inhibitors. Ammonium sulfate fractionation was therefore performed on the crude extract. ATP citrate lyase was recovered between 25 and 45% final concentration of ammonium sulfate. A protein fraction was also collected between 65 and 75%. A similar fraction, designated "Fraction K" has been reported to enhance the inactivation by ATP of acetyl-CoA carboxylase in a time and concentration dependent manner (40). The two fractions were incubated together in the absence or in the presence of cyclic-AMP, ATP-Mg and aminophylline. Again no change in the rate of inactivation of ATP citrate lyase could be seen.

D. DISCUSSION

The ability of insulin to lower the intracellular level of cyclic-AMP can be readily demonstrated in the liver of diabetic rats or in the liver of normal rats, first subjected to glucagon treatment (35,102). Many of the hepatic effects of insulin could be the result of its cyclic-AMP lowering effect. When glucagon is injected during refeeding of starved rats, lipogenesis is inhibited substantially (93). Similar effects can be seen with db-cyclic-AMP and theophylline. This treatment blocks the induction of fatty acid synthetase and acetyl-CoA carboxylase (93.96), malic enzyme (103), glucose-6-phosphate dehydrogenase (95) and glucokinase (94).

We have shown here that ATP citrate lyase is similarly affected. The induction of ATP citrate lyase activity that occurs during refeeding could be prevented in part by injection of a mixture of db-cyclic-AMP and theophylline or by injection of isobutylmethylxanthine, all known phosphodiesterase inhibitors. The high levels of cyclic-AMP in the starved state would then persist into the refed state. The removal of these drugs allows the activity of ATP citrate lyase to increase to high levels suggesting the return of cyclic-AMP concentration to low levels.

The prevention of the induction of ATP citrate lyase and the other lipogenic enzymes as a result of treatment with phosphodiesterase inhibitors or glucagon suggests an involvment of cyclic-AMP in the long-term control of

lipogenesis. This control of lipogenesis involves adaptive changes in the quantity of the lipogenic enzymes (10,49-56). Evidence have been presented that the reduction of fatty acid synthetase (93) and glucose-6-phosphate dehydrogenase (95) upon glucagon treatment is the result of decreased synthesis of enzyme, resulting in diminished quantity of enzyme protein. The implication from these results could be that cyclic-AMP has a repressing effect on the specific synthesis of the lipogenic enzymes (93), in analogy with its suggested stimulating effect on the synthesis of phosphoenolpyruvate carboxykinase. It is possible that cyclic-AMP produces the response indirectly. Prevention of glucokinase induction (11) would cause a drastic reduction in the rate of glycolysis which would lead to a decrease in the concentration of glycolytic intermediates. The reduction in supply of precursors would result in a lower rate of fatty acid synthesis and obviate the need for an increase in the lipogenic capacity. In addition, high concentration of cyclic-AMP would keep the triglyceride lipase in an active state, with concomittant high levels of fatty acyl-CoA.

The induction potential of high carbohydrate refeeding of starved rats varies with the fat content of the diet. Fat in the diet causes a decrease in the induction of lipogenesis and the lipogenic enzymes (101). The dampening effect of fat in the diet could be expected to act through an increase in the hepatic fatty acyl-CoA concentration. Only two physiologically important sites have been found to

be sensitive to end-product inhibition, namely acetyl-CoA carboxylase, where fatty acyl-CoA inhibition is competitive with citrate activation (13) and the citrate transport carrier system in the mitochondrial membrane (14). ATP citrate lyase is only moderately sensitive to long-chain acyl-CoA (50% inhibition at 0.1 mM concentration of palmitoyl-CoA)(104). This conclusion is further strengthened by studies on palmitoyl-CoA inhibition of citrate incorporation into long-chain fatty acids in chicken liver homogenate (15,105).

During these studies we observed that the amount of food consumed varied between the different groups of rats. A daily account was therefore kept of the food intake by each rat. It became apparent that the magnitude of the increase in ATP citrate lyase activity was dependent on the caloric consumption, as has been reported for the induction of glucose-6-phosphate dehydrogenase (57). When part of the carbohydrate is exchanged with fat the rats consumed less food, especially from the second day on, giving a smaller induction of ATP citrate lyase. Administration of db-cyclic-AMP and theophylline during refeeding of starved rats with a high carbohydrate, low-fat diet results in a further drop in the food consumption. The difference in consumption was particular impressive during the second and third day. Since the increase in ATP citrate lyase is sensitive to changes in the food consumption, the specific contribution of either fat or phosphodiesterase inhibitors

in preventing the induction of ATP citrate lyase is difficult to ascertain. It is possible that the effects are secondary to changes in the appetite.

While these studies could have been repeated with control of caloric intake by means of stomach intubation, it is likely that elucidation of the hormonal control of lipogenesis will be better achieved by studies on isolated liver cells, where nutritional conditions can be rigidly controlled.

Experiments on fatty acid synthesis in liver slices, in perfused liver preparations, and in isolated hepatocytes have indicated a role for cyclic-AMP in the short-term control. Klein and Weiser (37) demonstrated rapid inhibition of fatty acid synthesis by glucagon and that this was accompanied by a parallel reduction in acetyl-CoA carboxylase activity. Similar results have been obtained by incubation of liver slices with db-cyclic-AMP (38). A direct inhibition by db-cyclic-AMP of acetyl-CoA carboxylase in rat liver homogenate has also been shown (38). The mechanism by which cyclic-AMP affects the enzyme activity has yet to be established. It is noteworthy that purified acetyl-CoA carboxylase can be converted to an inactive phosphorylated form by an ATP-dependent protein-kinase (40,106). The reversal of this process is catalyzed by a Mg-dependent phosphatase fraction. The phosphorylation was, however, independent of cyclic-AMP. Several other lipogenic enzymes have been implicated to be under the control by

phosphorylation-dephosphorylation mechanisms (43-45), suggesting a coordinate control for the short-term regulation of the lipogenic enzymes. The fatty acid synthetase complex of pigeon liver can also be phosphorylated and inactivated by an ATP-dependent kinase and dephosphorylated and activated by a Mg-dependent phosphatase(45). Attempts to detect such a mechanism in rat or human liver have been unsuccessful (46,47). In contrast, the phosphorylation of L-type pyruvate kinase is mediated by a cyclic-AMP dependent protein kinase (44). Regulation of phosphofructokinase by phosphorylation-dephosphorylation has also been reported (43), but the data in this case are equivocal.

In our survey of regulatory control of ATP citrate lyase, an ammonium sulfate fraction of liver homogenate was incubated with ATP, in an experiment similar to that used by Carlson and Kim (40) to demonstrate phosphorylation of acetyl-CoA carboxylase. ATP citrate lyase activity assayed under optimal conditions was not affected by this incubation, nor did addition of cyclic-AMP to the system have an effect, suggesting that regulatory phosphorylation of the enzyme was unimportant. However such a modification might occur with a change in catalytic activity only detectable at suboptimal reaction conditions (44). It is also possible that some other functional properties of the enzyme are affected by phosphorylation (107).

After the conclusion of this part of our study, several reports appeared in the literature demonstrating structural

phosphorylation of ATP citrate lyase (108-110). Such phosphorylation occurs at a site different from the site which is phosphorylated during the catalytic route (111), and may be under hormonal influence. The role for this modification remains obscure, however, because structural phosphorylation does not change any of the kinetic or other properties of the enzyme. An equivalent structural phosphorylation has been been reported for acetyl-CoA carboxylase (112) which seems to be distinct from the phosphorylation correlated with the inactivation discussed above.

IV. PURIFICATION OF ATP CITRATE LYASE AND MALIC ENZYME

A. INTRODUCTION

The realization that citrate was required for fatty acid synthesis (113), led to the discovery of ATP citrate lyase by Srere (114), which catalyzes the following reaction (see Eqn.[1]):

$$\text{ATP} + \text{citrate} + \text{CoA} \rightleftharpoons \text{oxaloacetate} + \text{acetyl-CoA} + \text{ADP} + P_i \quad [1]$$

The enzyme has been found to occur in a variety of animal tissue (115) but is especially enriched in liver and adipose tissues, in keeping with its metabolic role in lipogenesis.

Despite the physiological significance of ATP citrate lyase, little is known about its structural properties, partly due to its instability during purification (20, 115-117). In the early investigation of Srere (115), the enzyme was partially purified from the soluble fraction of chicken liver and some of its properties studied. The fact that it can be induced by dietary manipulation was exploited by Inoue *et al* (20) in their purification of the enzyme from rat liver. These workers succeeded in enhancing the stability of the enzyme by the addition of 2-mercaptoethanol and $MgCl_2$ to the buffer media.

In our first attempts to purify ATP citrate lyase from rat liver, we were not able to reproduce the procedure of

Inoue *et al* (20). The addition of 2-mercaptoethanol and $MgCl_2$ did not stabilize the enzyme during the purification and our recovery of the enzyme after ion exchange chromatography on DEAE-cellulose was exceedingly small. We report here a modified purification procedure, where stabilization of the enzyme during the first stages is achieved by the inclusion of citrate in the buffer media. Furthermore, the success of the procedure depends mainly on its speed of operation.

From the same pooled rat livers, we were able to purify malic enzyme, which is much more stable than ATP citrate lyase. Malic enzyme, first shown by Ochoa *et al* (118) to catalyze the reaction (see Eqn.[4]):

$$\text{Malate} + \text{NADP}^+ \rightleftharpoons \text{pyruvate} + \text{NADPH} + \text{H}^+ \qquad [4]$$

is considered to be a lipogenic enzyme (49,119). It supplies 50% of the NADPH needed for fatty acid synthesis, the rest supplied by the pentose phosphate pathway. The enzyme has been purified from the livers of chicken (120), pigeon (121) and rat (122). The procedure presented here is simple and staightforward.

B. METHODS

1. Stabilization of ATP citrate lyase.

Our first objective was to find conditions where ATP citrate lyase is most stable. For use in these experiments, the enzyme was purified from Woodlyn/Wistar rats starved for 2 days and refed a high carbohydrate, fat-free diet for 3 days according to the procedure of Inoue *et al* (20). The ammonium sulfate-precipitated enzyme was dissolved in 10 mM Tris-HCl buffer pH 7.8 with or without 10 mM 2-mercapto-ethanol, 1 mM $MgCl_2$ and 0.1 mM EDTA and dialyzed overnight against the same buffer. The enzyme solution containing 2-mercaptoethanol, $MgCl_2$ and EDTA was divided into four parts. Either ATP, sodium citrate or sucrose was added to three of the solutions to final concentrations of 5 mM, 0.2 mM and 1 mM, respectively. Enzyme solutions were stored either at 0-5° or frozen, and the activity of ATP citrate lyase was followed over time.

2. Purification of ATP citrate lyase.

22 Sprague-Dawley rats were starved for 2 days and refed a high carbohydrate, fat-free diet for 3 days. The animals were decapitated and the livers were removed immediately and washed in ice-cold 10 mM Tris-HCl buffer pH 7.4, containing 0.2 M KCl, 1mM EDTA, 10 mM 2-mercaptoethanol and 5 mM sodium citrate. All subsequent operations were performed at 0-5°.

The livers (approx. 340 g.) were homogenized in 3 volumes of the above buffer with the aid of a Servall Omnimixer. The crude extract was centrifuged for 30 minutes (at 15,000xg with the aid of a RC2B refrigerated centrifuge for this and all subsequent centrifugations) and the supernatant was cleared by passing it through a layer of Kimwipes.

A freshly prepared 2% solution of protamine sulfate in the above buffer was added slowly to produce a final concentration of 0.2%. Stirring was continued for 10 minutes and the solution was centrifuged for 30 minutes.

To the clear supernatant, solid ammonium sulfate was added to 35% final concentration. The pH was kept between 7 and 8 by dropwise addition of concentrated NH_4OH. After stirring for 10 min. the extract was centrifuged for 30 minutes. The supernatant was used for the purification of malic enzyme. The precipitate was dissolved in the above buffer to a volume corresponding to one-third of the volume obtained after the protamine sulfate step.

A 25% solution of polyethylene glycol 6000 was prepared in the above buffer and added slowly to produce a final concentration of 5%. The extract was centrifuged for 30 minutes and the precipitate was discarded. Polyethylene glycol was then added to produce a final concentration of 10%. The solution was centrifuged for 20 minutes and the precipitate was dissolved in a minimum volume of 20 mM Tris-HCl buffer pH 7.4, containing 1 mM EDTA and 1 mM DTT.

The enzyme solution was desalted on a Sephadex G-25 coarse column (2.5 x 50 cm) equilibrated with the same buffer and then applied directly to a DEAE-Sephacel column (2.5 x 60 cm), which had been equilibrated with the same buffer. After washing the column with two column volumes of starting buffer, ATP citrate lyase was eluted by applying a linear gradient of 0 to 0.3 M KCl (a total gradient volume of 5 column volumes). Most active fractions were combined, concentrated by adding solid ammonium sulfate to 50% final concentration and centrifuged for 30 minutes.

The precipitate was dissolved in 20 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA and 1 mM DTT and applied to a Sephacryl S-200 superfine column (2.5 x 99 cm) equilibrated with the same buffer. Most active fractions were pooled.

A second DEAE-Sephacel step was usually necessary. Starting buffer was 20 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA, 1 mM DTT and 16 mM KCl. After washing the column a linear gradient from 16 mM KCl to 100 mM KCl was used to elute the enzyme.

Routinely 0.34 g. sucrose was added to each ml of enzyme solution and this was then frozen in portions.

3. Purification of malic enzyme.

The supernatant solution resulting from the ammonium sulfate precipitation of ATP citrate lyase was used for the purification of malic enzyme. The solution was adjusted to

50% final concentration of ammonium sulfate, stirred and centrifuged for 30 minutes. The precipitate was discarded and ammonium sulfate was added to the supernatant to 80% final concentration, stirred and centrifuged as before. The precipitate, containing malic enzyme activity was dissolved in a minimum volume of 20 mM Tris-HCl buffer pH 7.7 containing 2 mM 2-mercaptoethanol.

The extract was desalted on a Sephadex G-25 coarse column (5 x 60 cm) equilibrated with the same buffer, and applied directly to a DEAE-Sephacel column (5 x 45 cm) equilibrated and washed with the same buffer. After washing, the enzyme was eluted by applying a linear gradient of 5 column volumes of starting buffer and 150 mM Tris-HCl buffer pH 7.7 containing 2 mM 2-mercaptoethanol. Most active fractions were pooled and concentrated 10-fold with the aid of an Immersible Molecular Seperator (Millipore Corporation, Bedford, Massachusetts, U.S.A.).

The extract was finally applied to a Sephacryl S-200 superfine column (2.5 x 60 cm) equilibrated and run with 70 mM Tris-HCl buffer pH 7.7, containing 2 mM 2-mercaptoethanol.

The enzyme eluted immediately after the void volume, was pooled and stored in the cold as a precipitate in 75% ammonium sulfate.

C. RESULTS AND DISCUSSION

1. Isolation of ATP citrate lyase.

Table I summarizes the results of the survey of the stability of ATP citrate lyase under various conditions. Of the compounds tested at 0-5°, the best protection was afforded by citrate. Subsequently, we found that pH 7.4 gave a better recovery than pH 7.8 on the ion-exchange column, as was suggested by Inoue *et al* (20). Based on these findings we decided to develop a new procedure for the purification of ATP citrate lyase.

The homogenized crude liver extract was fractionated using a number of established techniques which utilize the different solubility characteristics of proteins. By the evening of the first day of purification, the extract can be applied to a DEAE-Sephacel column. This anion exchanger can withstand high flow rates and the elution of the enzyme is usually complete within 24 hours (Fig.4). The enzyme is finally applied to a Sephacryl S-200 superfine, where the enzyme elutes immediately after the void volume. In some cases the enzyme still contains impurities after this step which can be conveniently removed by a second DEAE-Sephacel column, using a slightly higher ionic strength and a more shallow gradient. The enzyme elutes in an apparent homogeneous state as judged by polyacrylamide gel electrophoresis in the presence of SDS. If the purification is done too slowly, an additional two bands with lower molecular weights appear in the gel electrophoresis pattern. These

TABLE I

Stability of ATP citrate lyase during storage

Temperature	Days of storage	Activity remaining (%)[1]				
		Additions				
		2-mercaptoethanol, $MgCl_2$ and EDTA				
		None		ATP	citrate	sucrose
0-5°	1	90	56	60	107	61
	3	20	6	12	80	12
	38	0	0	0	tr[2]	0
-20°	1	94	115	140	120	138
	3	19	22	34	72	55
	38	tr	tr	tr	27	51

[1] The activity after dialysis was set at 100%.
[2] tr = trace of activity.

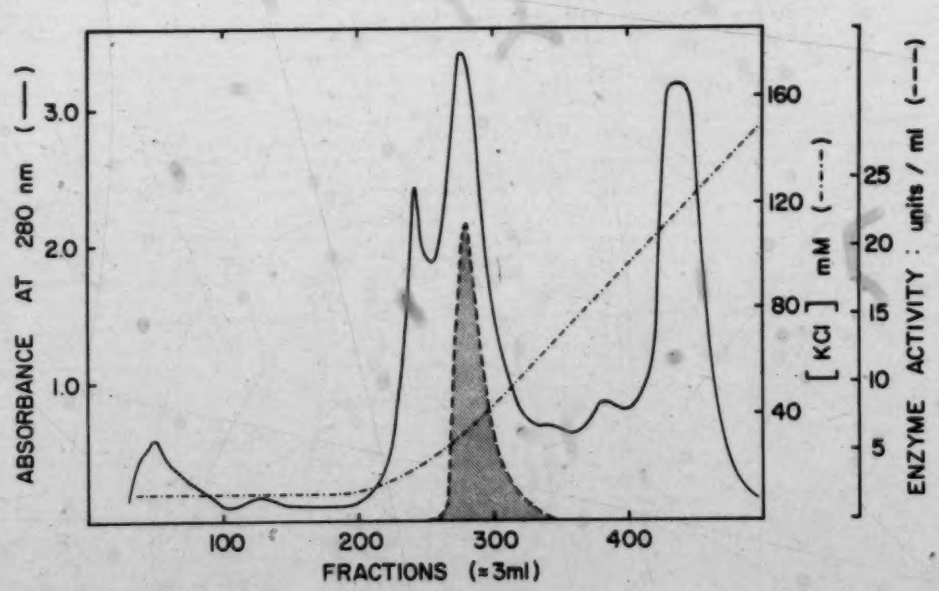

Fig.4. Chromatogram of ATP citrate lyase on first DEAE-Sephacel column (2.5 x 60 cm). After application of the protein, the column was washed with 2 column volumes of starting buffer; 20 mM Tris-HCl - 1 mM EDTA - 1 mM DTT, pH 7.4. Then a linear gradient of 0 to 0.3 M KCl in starting buffer (5 column volumes) was applied (at fraction 100) to elute the enzyme. ATP citrate lyase eluted at a salt concentration of 35 mM KCl.

polypeptides probably arise from the higher molecular weight band through limited proteolysis during the isolation, as discussed by Singh *et al* (123).

A summary of the purification procedure is given in Table II. The gel filtration is the slowest step and a fair amount of inactivation occurs. This is probably due to oxidation of the enzyme, since the activity can be almost completely restored by incubating the enzyme with DTT (124).

After the completion of this work a similar improved purification procedure was published by Linn and Srere (111). These investigators achieved good stabilization of the enzyme by including 10% glycerol and high concentrations of DTT in the buffers. This enables the recovery of more than 50% of the ATP citrate lyase activity.

University of Alberta

2. Storage of ATP citrate lyase.

Generally, storage protocols for ATP citrate lyase have been based on the principles of excluding air and including a reducing reagent such as DTT in the enzyme preparation (20,117,124,125). Despite these precautions the enzyme activity is lost within 1 to 3 weeks. We have tried to store the enzyme as an ammonium sulfate precipitate, but more than half of the activity is lost overnight at 0-5°. However, based on the experiments reported in Table I, we routinely add sucrose to the purified enzyme preparation and store it at -20°. We have been able to store the enzyme more than a

TABLE II

Purification of ATP citrate lyase

Step	Volume (ml)	Total protein (gram)	Total activity (units)	Specific activity[1]	Yield (%)
Crude extract	1030	39.5	3490	0.09	100
Protamine sulfate	1070	26.4	3410	0.13	98
Ammonium sulfate	357	5.5	2580	0.47	74
Polyethylene glycol	42	4.1	2320	0.57	66
DEAE-Sephacel	110	0.35	1180	3.4	34
Sephacryl S-200	61	0.13	330	2.5	9.5
Second DEAE-Sephacel	489	0.10	445	4.5	12.8

[1] Specific activity is expressed as units/mg protein.

year with very little loss in activity.

3. Properties of ATP citrate lyase.

When freshly purified enzyme is assayed the absorbance decreases linearly with time, the rate of decrease being proportional to enzyme concentration. On the other hand, enzyme preparations which have lost some activity either during purification or during storage are apparently activated during the assay (Fig. 5). The initial rate of decrease of absorbance, which is linear for 1 to 3 minutes, starts to increase and reaches finally a new linear rate. Fig. 6 shows that both the initial and the final rates are proportional to the enzyme concentration. The difference between the two rates can be reduced but not eliminated by prior incubation of the enzyme in the assay medium before adding CoA to initiate the reaction. A similar incubation was found to restore much of the ATP citrate lyase activity which had been lost during dialysis (126). The obvious candidate in the assay mixture to be responsible for this apparent activation is the reducing reagent.

4. Isolation and properties of malic enzyme.

The malic enzyme can be obtained from the same pooled rat livers that are used for the isolation of ATP citrate lyase. When ATP citrate lyase is precipitated with ammonium

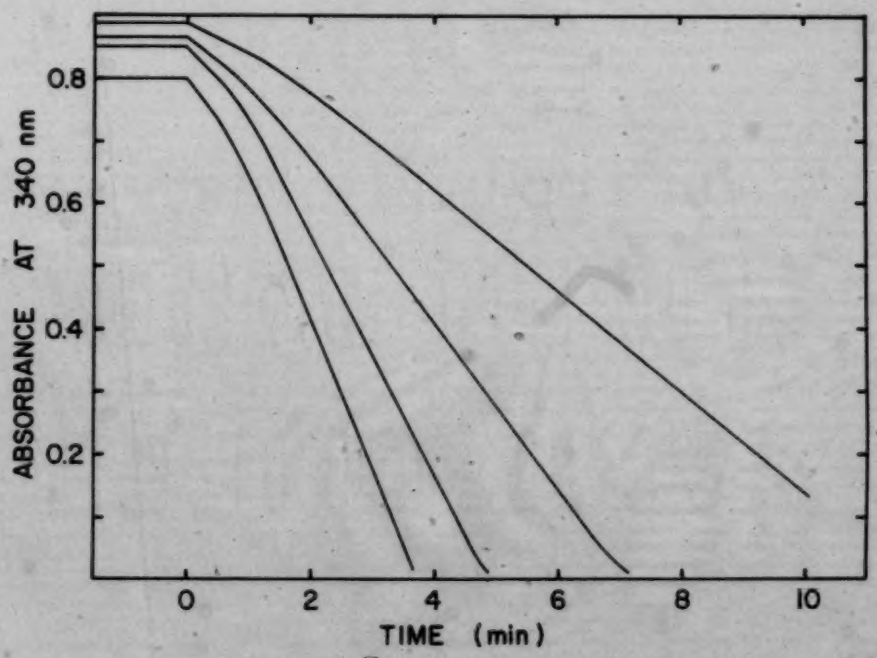

Fig.5. Effect of ATP citrate lyase concentration upon time course of NADH oxidation. ATP citrate lyase was assayed using the coupled enzymatic assay described in chapter II, where the time course is followed by the disappearance of NADH at 340 nm. 10 µl, 15 µl, 20 µl and 25 µl of ATP citrate lyase (0.79 mg/ml) were assayed and the reaction was started by the addition of CoA.

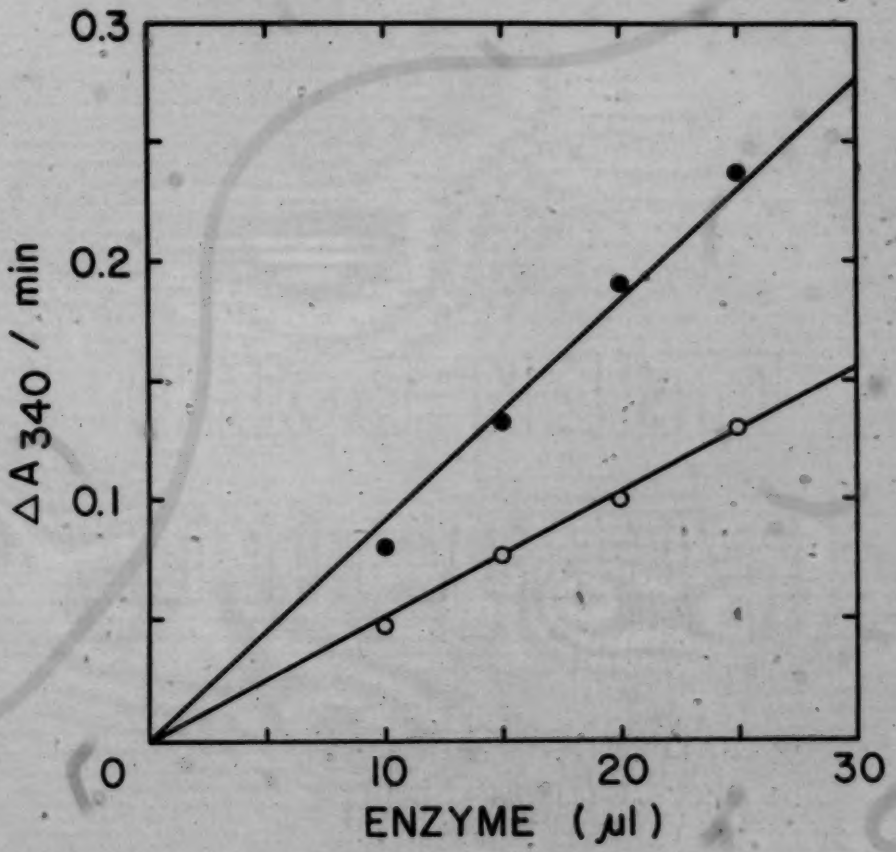

Fig.6. Effect of ATP citrate lyase concentration on the initial and the final rate of NADH oxidation. The initial (○) and the final rate of NADH oxidation (●) were determined from fig.5 and plotted against volume of ATP citrate lyase (0.79 mg/ml) added.

sulfate, the malic enzyme stays in solution and can successfully be fractionated between 50% and 80% final concentration of ammonium sulfate. After desalting, the extract is applied to a DEAE-Sephacel which removes most of the impurities (Fig. 7) and finally to a Sephacryl S-200 superfine column (Fig. 8) which gives an apparent homogeneous protein as determined by polyacrylamide gel electrophoresis in the presence of SDS. A summary of the purification is given in Table III.

In contrast to ATP citrate lyase, the malic enzyme is remarkably stable during its isolation and in the pure state. It can be stored for long periods of time as an ammonium sulfate precipitate without any loss in activity.

The spectrophotometric assay of malic enzyme, recording the appearance of NADPH at 340 nm, is linear with time and shows no lag.

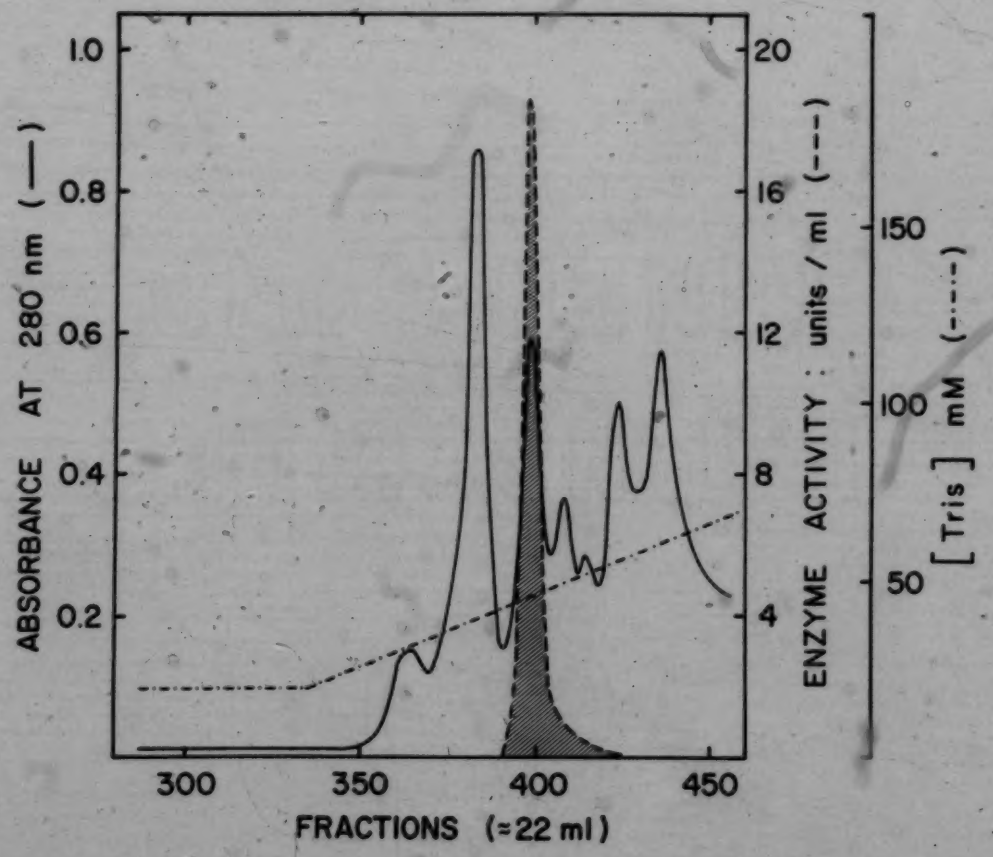

Fig.7. Chromatogram of malic enzyme on DEAE-Sephacel column (5 x 45 cm). After the sample was loaded, the column was washed with 7 column volumes (290 fractions) of 20 mM Tris-HCl - 2 mM 2-mercaptoethanol (pH 7.7), followed by a linear gradient of 20 mM to 150 mM Tris-HCl (2 mM 2-mercaptoethanol, pH 7.7) of 5 column volumes to elute the enzyme.

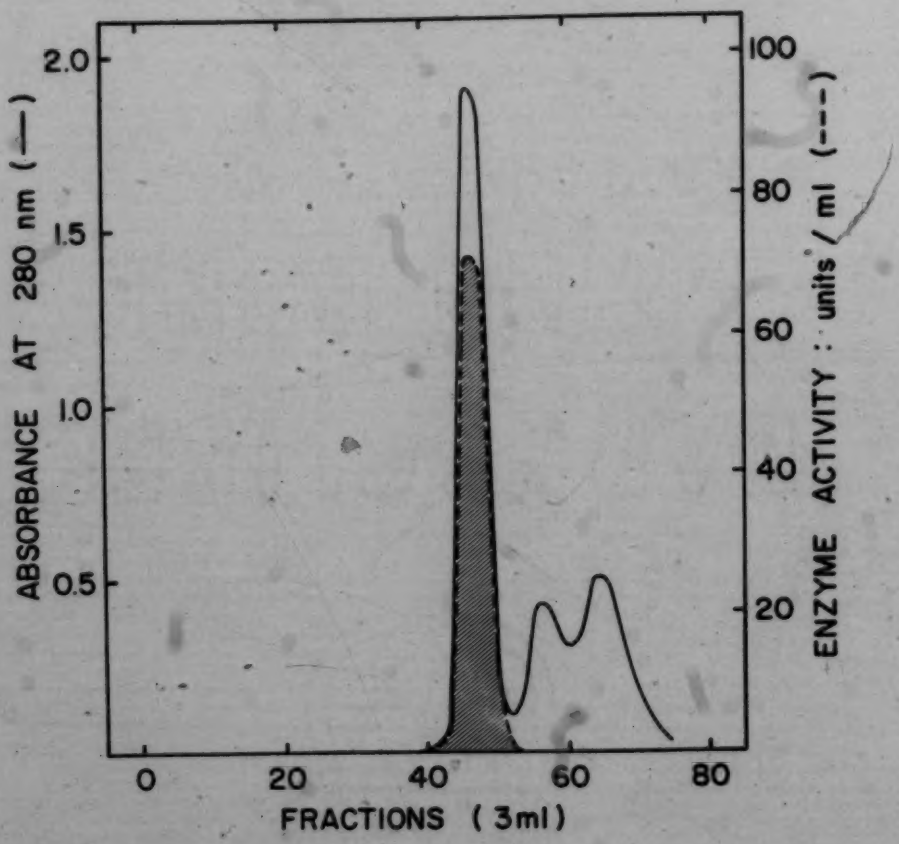

Fig.8. Chromatogram of malic enzyme on Sephacryl S-200 superfine column (2.5 x 60 cm). The column was equilibrated and eluted with 70 mM Tris-HCl buffer (pH 7.7), containing 2 mM 2-mercaptoethanol.

TABLE III

Purification of malic enzyme

Step	Volume (ml)	Total protein (gram)	Total activity (units)	Specific activity[1]	Yield (%)
Crude extract	1030	39.5	3757	0.1	100
Protamine sulfate	1070	26.4	3608	0.14	96
Ammonium sulfate	80	3.4	2737	0.8	73
DEAE-Sephacel	112	0.07	1772	25.3	47
Sephacryl S-200	19	0.03	1136	37.9	30

[1] Specific activity is expressed as units/mg protein.

V. A STABILIZING FACTOR FOR ATP CITRATE LYASE

A. INTRODUCTION

Phosphofructokinase activity in liver is subject to similar dietary adaptation as the lipogenic enzymes. Its induction has been shown to be the net result of both enhanced rate of protein synthesis and a decreased rate of protein degradation (50,128). Our attention was drawn to the phosphofructokinase system by the discovery by Dunaway and Segal (83) of a labile, dialyzable factor in rat liver supernatant, whose level fluctuates opposite to that of the degradation rate of phosphofructokinase and which can protect the enzyme against thermal and lysosomal inactivation *in vitro*. It was suggested that the factor might control the rate of degradation of phosphofructokinase *in vivo* by shifting the equilibrium between enzyme conformers to a more proteolytically resistant form.

The phosphofructokinase stabilizing factor was purified from rat liver to apparent homogeneity (83). The factor was reported to have a molecular weight of approximately 3,500 and was thought to be a peptide, based on its ultraviolet spectrum, susceptibility to pronase and correspondence with ninhydrin-positive material on gel filtration and paper electrophoresis. Amino acid composition (glu:gly:cys; 1:1:1) suggested the presence of glutathione or a derivative in the factor preparation (83).

Because of the parallels in the dietary adaptation pattern between phosphofructokinase and ATP citrate lyase, we thought it to be an attractive possibility that the factor might affect the stability of the latter enzyme. We partially purified the stabilizing factor according to Dunaway and Segal (83) and the experiments reported in this chapter show that the factor exerts similar protective effects on ATP citrate lyase.

Together with the earlier work on phosphofructokinase, these experiments suggested the exciting concept that the stabilizing factor might be a pleiotropic mediator, regulating (at least) lipogenic enzyme turnover in response to insulin stimulation. For these reasons, the effect of the factor was also tested on malic enzyme, another adaptable lipogenic enzyme.

B. METHODS

1. Partial purification of ATP citrate lyase.

Most of these experiments were performed before the development of our standard purification procedure (see chapter IV) and the enzyme preparation used was obtained as follows.

200 gram Woodlyn/Wistar rats were starved for two days and then refed a high carbohydrate, fat-free diet for three days. ATP citrate lyase was isolated from the liver and partially purified according to a procedure adopted from Inoue *et al* (20) and modified as follows. Livers were homogenized in three volumes of 10 mM Tris-HCl buffer pH 7.4, containing 10 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM sodium citrate and 0.2 M KCl. Following centrifugation at 15,000xg for 30 minutes, 2% protamine sulfate in the same buffer was added to give a final concentration of 0.2%. The enzyme was precipitated from the supernatant with 45% final concentration of ammonium sulfate. The precipitate was dissolved in the above buffer modified to contain only 20 mM KCl and having a pH of 7.8 and then dialyzed against the same buffer. DEAE-cellulose was equilibrated with dialysis buffer and elution was carried out by using a continous gradient of dialysis buffer adjusted to pH 7.4 and to a final concentration of KCl of 0.4 M. Active fractions were pooled and concentrated with 45% ammonium sulfate. The precipitate was dissolved in homogenizing buffer and applied to a column of Sephadex G-200 equilibrated with the same

buffer. Fractions with a specific activity over 1.0 units/mg were pooled and sucrose was added (0.34 gram sucrose to 1.0 ml of solution). The enzyme so obtained was stored at -20° until used. This partially purified enzyme was used for the studies concerning thermal stability and treatment with trypsin and chymotrypsin.

2. Complete purification of ATP citrate lyase.

The ATP citrate lyase preparation used for the studies concerning lysosomal degradation was isolated and purified from rat liver as described in chapter IV.

3. Preparation of the stabilizing factor.

For the early experiments described in this chapter, the stabilizing factor was prepared essentially according to Dunaway and Segal (83). Woodlyn/Wistar rats were starved 4 days and then refed a high carbohydrate, fat-free diet for one day. Liver were homogenized in 50 mM Tris-HCl buffer pH 8.3, containing 250 mM glucose and 10 mM 2-mercaptoethanol. The 90% ammonium sulfate supernatant was extensively dialyzed using Spectrapor 6 membrane tubing (mol.weight cut off: 2,000). The solution was then concentrated with an Amicon High-Performance Thin-Channel Ultrafiltration System using a UM2 Diaflo membrane (mol.weight cut off: 1,000). This preparation of the stabilizing factor was used to

investigate its effect on the thermal inactivation of ATP citrate lyase and its susceptibility to trypsin and chymotrypsin. The stabilizing factor was subjected to gel filtration on a column of Sephadex G-25 fine (1.5 x 85 cm) pooled and concentrated by lyophilization before being used in the studies with lysosomal extract.

4. Thermal inactivation of ATP citrate lyase.

Thermal inactivation of ATP citrate lyase was performed in a Temp-Blok Module Heater set at 37°. The time needed for 50% inactivation was calculated using linear regression analysis on semi-logarithmic plots.

5. Tryptic and chymotryptic treatment of ATP citrate lyase.

Tryptic digestion of ATP citrate lyase was done according to Singh *et al* (123). To 1 mg of enzyme in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.2 M KCl was added 0.01 mg TPCK-treated trypsin and after 20 minutes incubation at 25°, 0.4 µg TLCK was added to stop the reaction. Trypsin and TLCK were dissolved in 0.1 M Tris-HCl buffer pH 7.4. In the control sample, buffer was added instead of trypsin.

Chymotrypsin digestion was done similarly. To 1 mg enzyme in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.2 M KCl was added 0.05 mg

chymotrypsin. The remaining activity of ATP citrate lyase was determined at time intervals, and finally the inactivated enzyme was analyzed by SDS gel electrophoresis.

6. Preparation of lysosomal extract.

Lysosomal extracts were obtained by the procedure of Ragab *et al* (80). Sprague-Dawley rats weighing about 350 gram were used. The "lysosomal composite fraction" suspended in 0.1 M Tris-HCl pH 7.0, containing 1 mM EDTA, 10 mM DTT and 0.25 M sucrose, was freeze-thawed 10 times and then centrifuged at 100,000xg for 60 minutes. The resulting supernatant, containing a 15-fold enrichment in the specific activity of acid phosphatase (80), was used as lysosomal extract.

7. Protein determination.

Concentration of stabilizing factor was estimated from the absorbance at 190 nm following exhaustive dialysis against water (83).

8. Stability experiments with malic enzyme.

Malic enzyme was dialyzed overnight against 20 mM sodium phosphate buffer pH 7.7, containing 2 mM 2-mercapto-ethanol. 15 µl enzyme (28 µg) was mixed with 40 µl 20 mM

sodium phosphate buffer pH 8.0 or stabilizing factor (12.0 mM) and 10 µl of 1.0 M sodium phosphate, for pH 6.0-7.7 or 1.0 M Tris-HCl for pH 8.0-10.0 was added to produce the desired pH. For pH studies, the mixture was incubated at 37° and for temperature studies, the pH of the mixture was 7.7. Samples were removed at time intervals for assay of remaining activity.

C. RESULTS

1. Thermal inactivation of ATP citrate lyase.

The stability of ATP citrate lyase at 37° depended on the pH, as shown in Fig. 9. At a protein concentration of 1 mg/ml there was no appreciable loss of enzyme activity within 2 hours at pH 7.4, whereas at pH 8.3 the enzyme rapidly lost its activity.

The stabilizing factor described by Dunaway and Segal (83) was purified up to and including the dialysis step. When the factor was added in increasing amount to ATP citrate lyase and the mixture was incubated at 37° and pH 8.3, ATP citrate lyase was found to be protected against thermal inactivation (Fig.10). A control sample, in which equivalent amounts of dialysate replaced the factor, showed no stabilization. The observed stabilization of ATP citrate lyase is a function of the concentration of factor(Fig.11).

As indicated earlier, Dunaway and Segal (83) have reported an unusual amino acid composition for the stabilizing factor. The ratio of glutamate:glycine:1/2-cystine was found to be approximately 1:1:1, with only traces of other amino acids. At this stage we wanted to ascertain that our factor preparation was identical to the one described for phosphofructokinase. To confirm this our factor, purified up to and including the dialysis step, was subjected to acid hydrolysis with or without prior performic acid oxidation. The results agree with those of Dunaway and Segal (83), indicating approximately equal amounts of

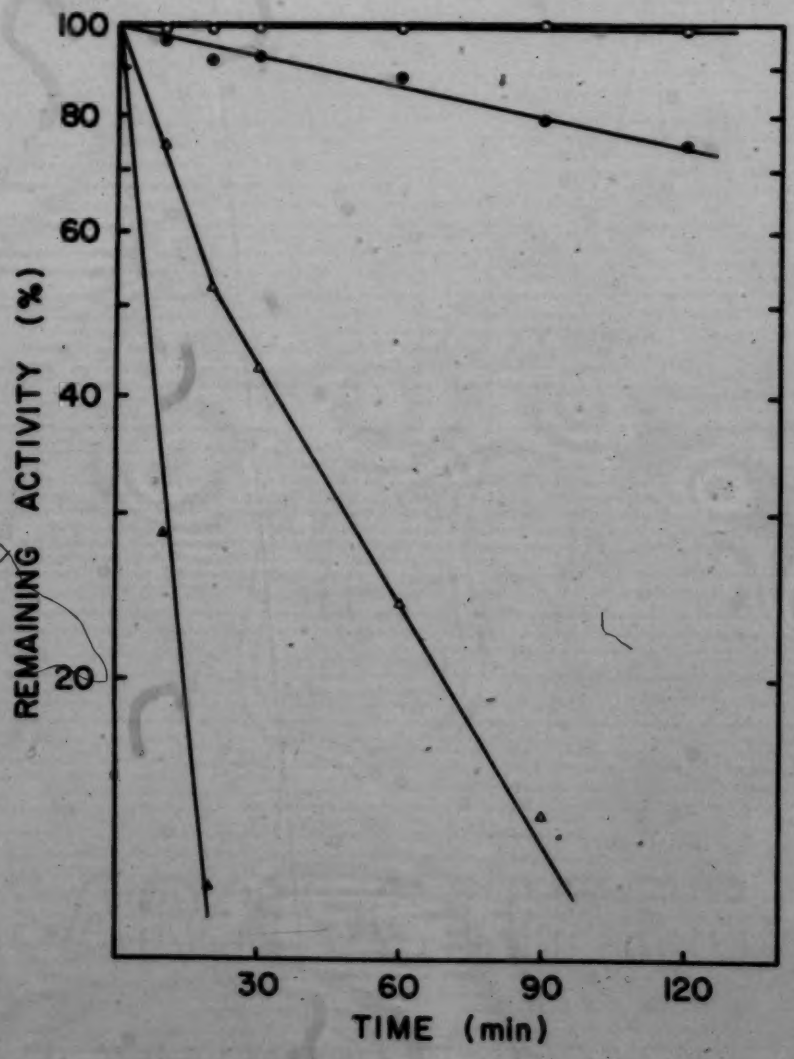

Fig.9. The effect of pH on the thermal inactivation of ATP citrate lyase. ATP citrate lyase was diluted to a concentration of 1 mg/ml in 50 mM Tris-HCl, containing 250 mM glucose and 10 mM 2-mercaptoethanol. Incubation was at 37° and the final pH was 7.4 (○), 7.7 (●), 8.0 (△) and 8.3 (▲).

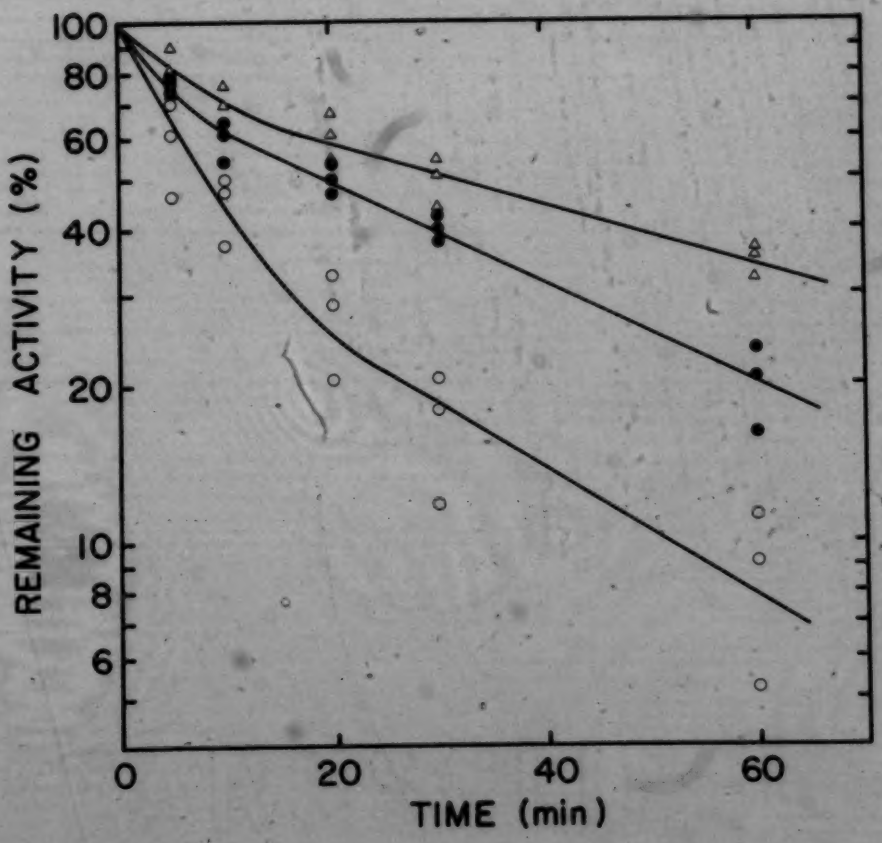

Fig.10. Effect of stabilizing factor on the thermal inactivation of ATP citrate lyase. 75 µl ATP citrate lyase (2.2 mg/ml) was mixed with 75 µl 50 mM Tris-HCl buffer, pH 8.3, containing 250 mM glucose and 10 mM 2-mercaptoethanol plus or minus stabilizing factor (89.7 µg/ml). (○) Enzyme alone, (●) plus 50 µl stabilizing factor, (△) plus 75 µl stabilizing factor. An identical experiment with 25 µl stabilizing factor was omitted from the figure for the sake of clarity. At each concentration the rate of degradation in three separate mixtures were determined.

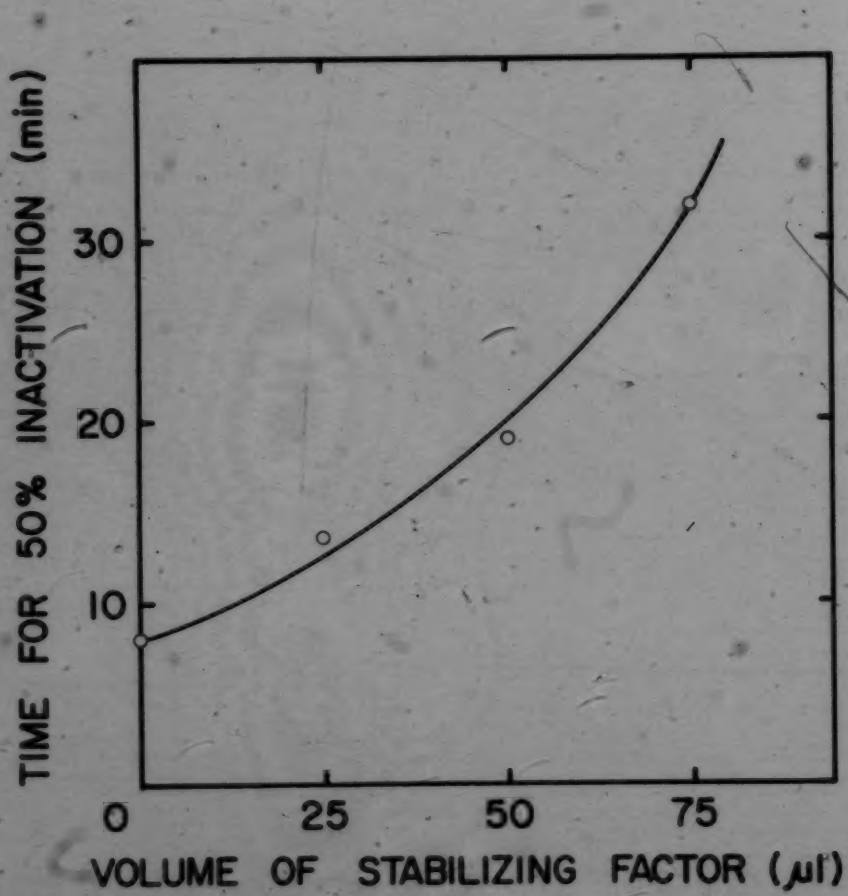

Fig.11. The dependence of the stabilizing effect on the concentration of stabilizing factor. The time needed for 50% inactivation from fig.10 was plotted against volume of stabilizing factor.

glutamate, glycine and halfcystine, plus small (<5%) traces of other ninhydrin-positive material. However, as will be reported in chapter VI, analysis of a further purified factor preparation gave a somewhat different picture.

2. Treatment of ATP citrate lyase by various proteases.

a. Trypsin treatment.

Recently, Singh *et al* (123) demonstrated that ATP citrate lyase, under controlled conditions, could be nicked by trypsin treatment giving rise to a fully active but relatively unstable derivative. This process appears to involve cleavage of the 110,000 mol. weight subunit to produce two smaller fragments. It was of obvious interest to determine the possible relationship of proteolysis to the effect of the stabilizing factor on the enzyme. Treatment of ATP citrate lyase with trypsin resulted in the conversion of the major peptide to two smaller fragments (Fig.12), a process accompanied by no detectable loss of activity. When the nicked enzyme was incubated at 37° and pH 8.3, it was rapidly inactivated with a t1/2 of 3.8 minutes (Fig.13), confirming the increased lability described by Singh *et al* (123). Untreated enzyme showed a t1/2 of 8 minutes (Fig.10). When the nicked enzyme was incubated in the presence of the stabilizing factor, we observed a degree of protection from inactivation comparable to that seen with native enzyme. If the stabilizing factor is present during the trypsin

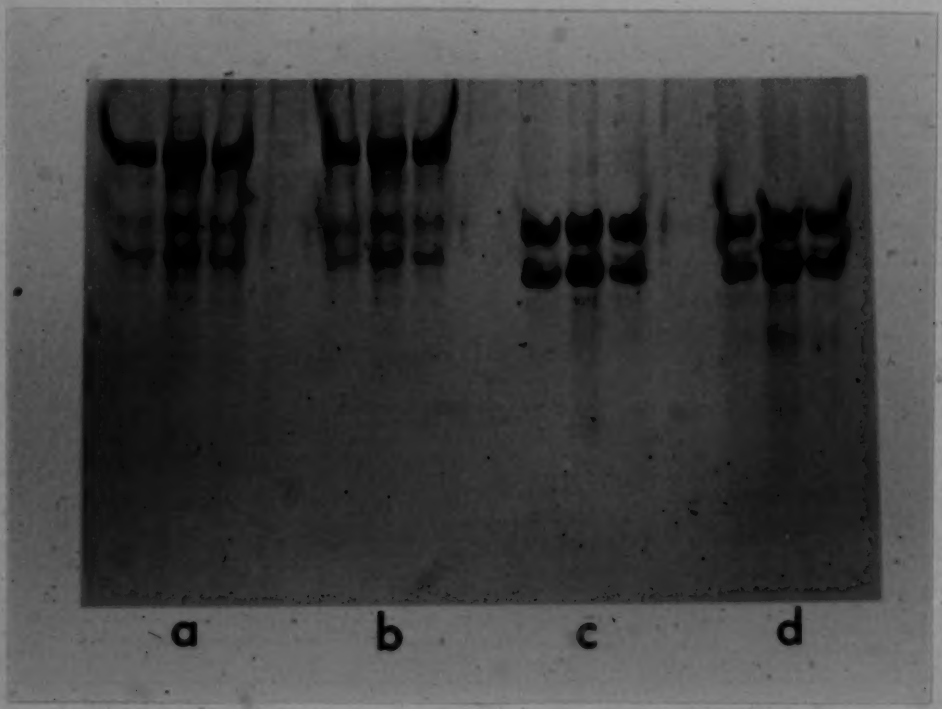

Fig.12. SDS gel electrophoresis of native and trypsin trated ATP citrate lyase. 5µg (outer tracks in each set of three) or 10 µg (middle track) of ATP citrate lyase were electrophoresed on 3 mm slabs in the presence of 0.1% SDS and the protein was stained with Coomassie blue. The samples applied were: a) native enzyme; b) enzyme carried through proteolysis procedure but without trypsin addition; c) trypsin treated enzyme; and d) enzyme treated with trypsin in the presence of stabilizing factor. (19 µg of factor to 1 mg enzyme).

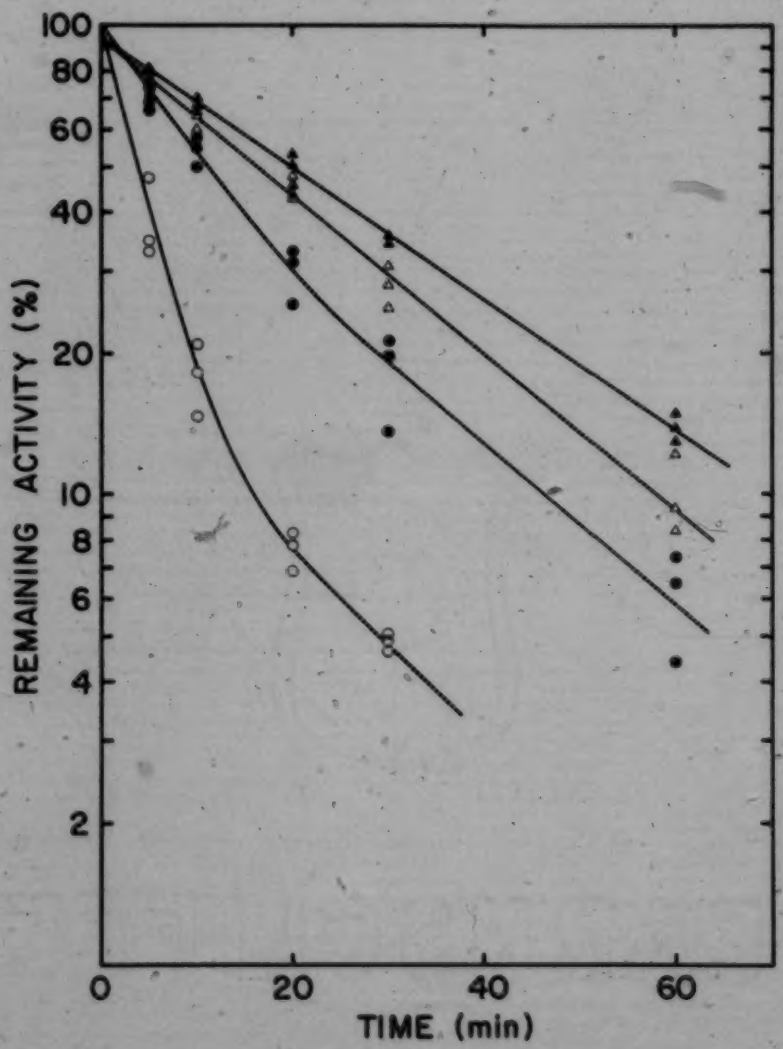

Fig. 13. The effect of stabilizing factor on nicked and unnicked ATP citrate lyase. The rate of degradation was measured in a mixture of 1 mg trypsin-treated enzyme minus (○) or plus (●) 19 µg stabilizing factor at 37°. (△) Enzyme (1 mg) treated with trypsin in the presence of 19 µg stabilizing factor. (▲) control in which enzyme was handled identically except that trypsin was omitted from the pretreatment. Each experiment was done in triplicate.

treatment, no change in the pattern of proteolysis was detected by SDS gel electrophoresis (Fig.12); nevertheless, the resultant product is protected to a significantly greater extent than the enzyme, which had been nicked in the absence of factor. These results suggest that while the factor apparently does not prevent the major endoproteolytic cleavage, it may protect against proteolysis near the end of a polypeptide chain, which may be accompanied by further reduction in stability but little change in size.

b. Chymotrypsin treatment.

Treatment of ATP citrate lyase with chymotrypsin leads to complete loss of activity. In addition to the two major polypeptides a number of additional protein bands with lower molecular weight was observed on SDS gel electrophoresis (Fig.14). Similar results were obtained by Singh and co-workers (123). The stabilizing factor had no effect on the inactivation and its presence had no effect on the pattern of protein bands on SDS gel electrophoresis.

c. Lysosomal extract.

Table IV shows the result of an experiment testing the effect of the stabilizing factor on the susceptibility of ATP citrate lyase to a lysosomal extract. When ATP citrate lyase was incubated with a lysosomal extract at pH 7.4 and

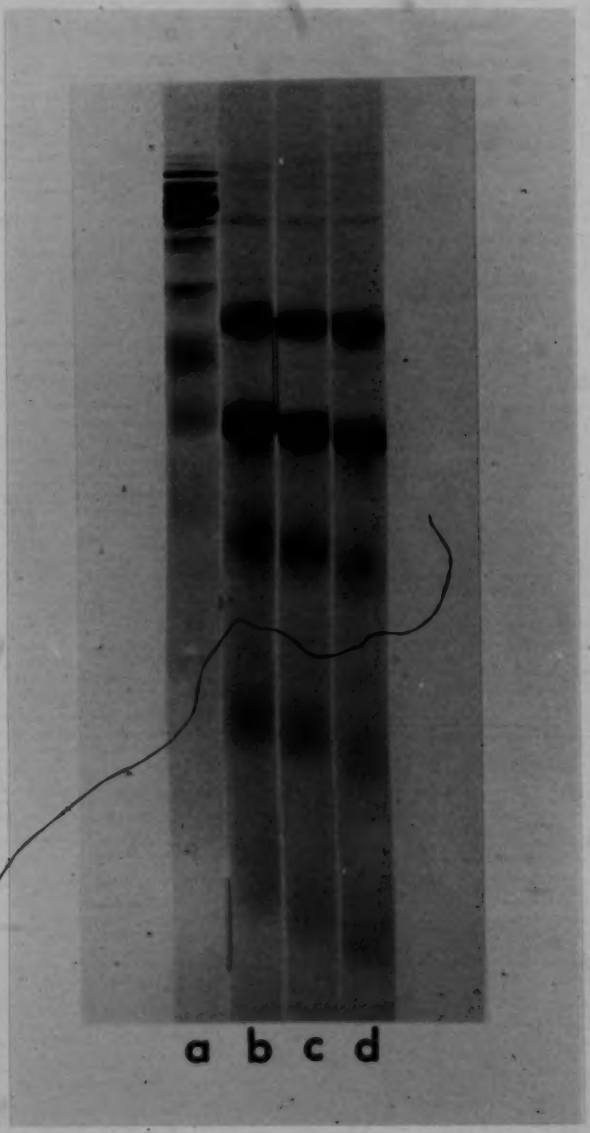

Fig.14. The effect of stabilizing factor on chymotrypsin digestion of ATP citrate lyase. ATP citrate lyase was treated with chymotrypsin in the absence or presence of 30 μg stabilizing factor/mg enzyme. The activity of ATP citrate lyase was followed and at time intervals samples were removed and applied to SDS gel electrophoresis. (a) Enzyme alone after 40 min. (b) enzyme and chymotrypsin after 40 min, and (c) after 90 min., (d) enzyme, chymotrypsin and stabilizing factor after 40 min.

TABLE IV

Protection by stabilizing factor against lysosomal inactivation of ATP citrate lyase at pH 7.4 and 37°

Stabilizing factor	k_d $(min)^{-1}$		
	(1) with lysosomes	(2) without lysosomes	net attributable to lysosomes: (1) - (2)
Absent	0.070	0.0153	0.055
Present	0.0034	0.0027	0.001

25 µl ATP citrate lyase (23 µg) was incubated with or without 50 µl lysosomal extract (108 µg protein), with or without 50 µl stabilizing factor (100 nmoles amine) at 37° and pH 7.4. First order rate constants (k_d) were calculated from the linear semilogarithmic plots of remaining activity versus time.

37°, its rate of inactivation was 4.5 times greater than in the absence of lysosomal extract. The stabilizing factor reduced this increase by 98%. Thus the factor provided effective protection of the enzyme from inactivation by a lysosomal extract, a result similar to that observed with phosphofructokinase (83).

In order to investigate the fate of ATP citrate lyase during lysosomal inactivation, a similar experiment was performed with the amount of lysosomal extract lowered 30-fold. During the course of the experiment samples were removed for assay of remaining activity (Fig.15) and for analysis on gel electrophoresis in the presence of SDS (Fig.16).

In the presence of stabilizing factor and lysosomal constituents, ATP citrate lyase (Fig.16a) was quickly nicked (Fig.16f-i) to form two associated fragments with full enzymatic activity. More general proteolysis and inactivation that occured in the absence of stabilizing factor (Fig.16b-e) was retarded. On the other hand the initial nicking that occured in the presence of stabilizing factor was much faster than that in the absence of factor. During the 4 hours incubation period, the controls showed very little inactivation (activities remaining after 4 hours were 84% and 97% for enzyme alone and enzyme plus stabilizing factor, respectively) and no change in the gel pattern could be seen. The data therefore suggests that the association of the factor with ATP citrate lyase promotes a

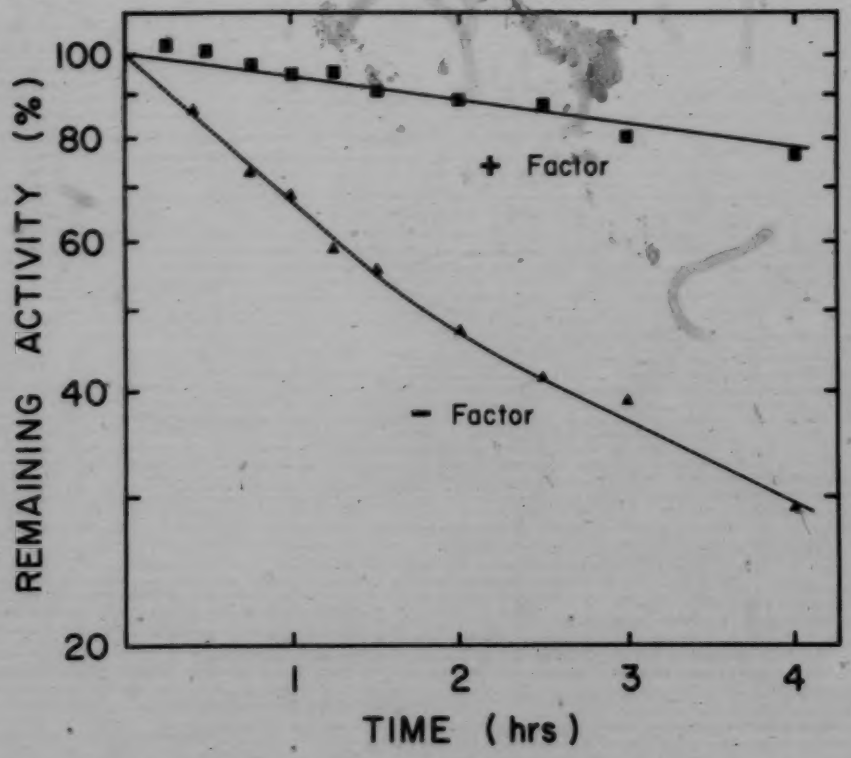

Fig.15. Effect of stabilizing factor on lysosomal inactivation of ATP citrate lyase. 1 mg ATP citrate lyase was incubated with 0.15 mg lysosomal extract, with or without 48 µg stabilizing factor at pH 7.4 and 37°. Samples were removed at intervals for assay of remaining activity and for application to SDS gel electrophoresis.

Fig. 16. SDS gel electrophoresis of ATP citrate lyase inactivated for various times by lysosomal extract in the absence and presence of stabilizing factor. 88 µg protein was applied in each well. Samples from left to right are as follows: a) enzyme alone, (b-e) 0, 1, 2 and 4 hours in the absence of stabilizing factor, (f-i) 0, 1, 2 and 4 hours in the presence of stabilizing factor.

preferential proteolytic attack with the production of a stable and active enzyme species, that is resistant to further degradation.

3. Stability studies of malic enzyme.

A series of experiments were initiated to determine the conditions where thermal inactivation of malic enzyme proceeded at a reasonable speed. Malic enzyme turned out to be a remarkably stable enzyme. At 37° the enzyme was very stable between pH 6.0 and pH 10.0 (Table V), although at pH's below 7.0 some slight inactivation could be observed. When the mixture was held at pH 7.7 and the temperature varied, a rapid inactivation occured above 50°. When the stabilizing factor was included, we could not observe any stabilizing effect.

Malic enzyme also showed surprising tolerance to the presence of proteases; concentrations of lysosomal extract that quickly degraded ATP citrate lyase had no effect on the activity of malic enzyme, and no proteolysis could be detected by subsequent electrophoretic analysis (not shown). However, trypsin treatment resulted in a rapid loss of about 50% of the activity. At pH 7.7 and 37°, the 50% level was reached in less than 5 minutes with 10% trypsin to malic enzyme (weight by weight). Progressively less trypsin only resulted in longer times needed to reach this level. Many attempts to demonstrate an effect of the stabilizing factor on the proteolytic susceptibility of malic enzyme were without success.

TABLE V

Effect of heat and pH on malic enzyme

Temperature	pH	Activity remaining after 30 min. (%)[1] - factor	Activity remaining after 30 min. (%)[1] + factor
37°	6.0	82	nd[2]
37°	6.6	82	nd
37°	7.0	96	nd
37°	7.7	112	nd
37°	8.0	94	nd
37°	9.0	101	nd
37°	10.0	107	nd
45°	7.7	100	nd
50°	7.7	92	nd
51°	7.7	87	nd
52°	7.7	68	65
55°	7.7	13	15

[1] The experimental details are described under "Methods". The values shown are from a single representative experiment.
[2] nd = not determined.

D. DISCUSSION

The results presented here indicate that a factor present in liver has the ability to confer thermal stability on ATP citrate lyase and the factor in question is very likely identical to one previously shown to stabilize phosphofructokinase against thermal and lysosomal inactivation (83). The demonstration that the factor has a very dramatic protecting effect on ATP citrate lyase against inactivation by a lysosomal extract lends further credibility to an important physiological role for the stabilizing factor.

It was suggested by Dunaway and Segal (83) that the basis of the effects of the interaction of the stabilizing factor with phosphofructokinase may be promotion of a shift to a conformational state of the enzyme which is less susceptible to thermal and lysosomal inactivation. The time course of changes in the level of the factor and of phosphofructokinase further supports a role for the factor as a regulator of the degradation rate of this lipogenic enzyme (50,83,128).

One important question is whether the degree of proteolytic and thermal susceptibility is related to the half-life of a protein. A general correlation has been found where short-lived enzymes are less resistent to inactivation by heat (73). Similar conclusions were obtained when melting curves were measured and compared with degradation rates for nine different intracellular enzymes (129). This study led

to the proposal that protein turnover is thermodynamically controlled such that susceptibility for degradation depends on the equilibrium of conformational states that a protein can assume. These conformers could differ from each other by being more or less unfolded, implying that the more unfolded a protein is (i.e., a large unfolding equilibrium constant) the higher the rate of degradation. With regard to the pathway of degradation, Ballard suggested (63) that the initial step in the degradation of phosphoenolpyruvate carboxykinase is a thermal denaturation to be followed by proteolysis. In contrast, Segal (130) concluded that the degradation of alanine aminotransferase does not depend on prior thermal denaturation.

The relevance of thermal inactivation of ATP citrate lyase to its rate of degradation is further complicated by the observation that the activity is highly dependent on the reduction state of the enzyme (131). The pH dependence of the thermal inactivation of ATP citrate lyase (Fig.9) suggests that the loss of activity is related to an oxidation of particularly vulnerable sulfhydryl groups on the enzyme. Experiments related to this aspect, together with the molecular mechanism of the stabilization process exerted by the factor, are further discussed in chapter VII.

The lysosomes occupy a central position in all discussions of protein degradation (78). These organelles contain the majority of the proteolytic activity in the cell and although practically all of the hydrolases exert maximum

activity in the acid region, amidases have been identified in the lysosomes which are most active at neutral pH (132,133). A good correlation has been observed between proteins' susceptibility to degradation by lysosomal extract and their half-lives *in vivo* (76,77). These studies, as well as the experiments by Dunaway and Segal (83) on the effect of the stabilizing factor on phosphofructokinase, were performed in slightly acid conditions. Our experiments were performed at pH 7.4, where the acid proteases of the lysosomal extract should show very little activity. This suggests to us that ATP citrate lyase is either extremely susceptible to proteolytic activity or that neutral proteases are present in the lysosomal extract. Interestingly, acetyl-CoA carboxylase has also been found to be degraded by a lysosomal extract at neutral pH (82).

The main problem in assigning a role for the lysosomes in the degradation of individual proteins is the mechanism by which these proteins are selected for removal. It has been suggested that proteins are actively transported into and out of the lysosomes and once inside the selection is made based on their susceptibility to proteolytic attack in the acid environment (78). While this could explain the apparent energy requirement noted for protein degradation (134), the idea of large proteins mobility through the lysosomal membrane is not generally accepted.

Another aspect of the mechanism of protein degradation is the discovery of soluble proteases specific for certain

groups of enzymes (79). The existence of these proteases increases the flexibility of the proteolytic machinery in such a way that groups of related proteins could be selected for degradation dependent on the presence or absence of ligands such as cofactors, substrates or products, which can stabilize or labilize the proteins. The stabilizing factor could then be regarded as another ligand, specifically regulating the turnover of lipogenic enzymes. The factor has now been shown to affect phosphofructokinase and ATP citrate lyase and it is very plausible that this group will include other lipogenic enzymes.

Another intriguing result that has come out of this work and the results presented by Singh *et al* (123) is the limited proteolytic cleavage (nicking) caused by each of trypsin, chymotrypsin and lysosomal extract. The initial step in the degradation pathway for ATP citrate lyase could be nicking to form a modified enzyme which is more labile (123). The stabilizing factor did not protect against nicking, but actually enhanced the rate of the initial cleavage with protection against further inactivation by lysosomal extract. The role of the factor in the induction of ATP citrate lyase could then be to decrease the rate of degradation and thereby assist in increasing the amount of the enzyme to supranormal levels. The presence of the stabilizing factor would also allow for an increase in the rate of modification of the enzyme by nicking by some soluble intracellular protease. This nicked enzyme, once the

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stimulus for induction is removed and the factor level is at low levels, would be removed quickly to return the amount of the enzyme to basal levels. While these ideas are highly speculative, some support has been found in that nicked enzyme is more labile than unnicked enzyme (123). Furthermore a correlation has been found between *in vivo* half-lives and susceptibility to proteolytic attack by specific proteases, such as trypsin and chymotrypsin, but not with non-specific proteases, such as subtilisin and pronase (135).

Results on hand suggest that the stabilizing factor may be specific for phosphofructokinase and ATP citrate lyase, since it does not affect glucokinase or pyruvate kinase (83) or malic enzyme as shown in this work.

The failure of the factor to stabilize malic enzyme may correlate with the observation that the degradation rate of this enzyme in liver is not reduced following a cycle of starvation and refeeding (120). Although malic enzyme is viewed as a lipogenic enzyme, partly responsible for generation of NADPH to be used for the synthesis of fatty acids, many observations suggest that the enzyme may participate in other roles as well (174). The hepatic enzyme is induced upon depletion of the protein in the diet, a condition not correlated with an increased rate of lipogenesis. If malic enzyme is involved in alternative functions (175), it is not surprising that it is not affected by the stabilizing factor which might be very specific in the regulation of

lipogenesis.

Further experiments to elucidate the chemical nature of the stabilizing factor are reported in subsequent chapters of this thesis.

VI. PURIFICATION OF THE STABILIZING FACTOR

A. INTRODUCTION

The stabilizing factor, first discovered by Dunaway and Segal (83), has now been shown to decrease the *in vitro* lability of two lipogenic enzymes, namely phosphofructokinase (83) and ATP citrate lyase (chapter V), against both heat and a lysosomal extract. The possibility that the factor may play an important role as a regulator of enzyme degradation was considerably increased by the finding that its level in the liver responded to the same stimulus and in the same direction as phosphofructokinase (83,128) and ATP citrate lyase (10).

Dunaway and Segal (83) isolated the stabilizing factor in the presence of 0.25 M glucose and the preparation was judged to be pure based on (1) the appearance of a symmetrical peak after the final gel filtration on Sephadex G-50, (2) the presence of only one band on SDS polyacrylamide gel electrophoresis after staining with Coomassie blue, and (3) the occurence of one ninhydrin-positive spot on paper electrophoresis. The amino acid composition suggested the presence of glutathione, but the factor was thought not to be glutathione *per se*, based on its ineffectiveness as a stabilizing agent of phosphofructokinase. Furthermore, commercial glutathione separated from the stabilizing activity on Sephadex G-50, and it was shown that glutathione taken through the purification procedure was removed at the

dialysis step.

In order to assess the physiological role of the stabilizing factor and its interaction with ATP citrate lyase, we deemed it essential to clarify the chemical nature of the factor. This chapter is concerned with the further purification and characterization of the stabilizing factor.

B. METHODS

1. Preparation of stabilizing factor in glucose medium.

The stabilizing factor was prepared essentially according to Dunaway and Segal (83) as described in chapter V. The 250 mM glucose solution was buffered either with 50 mM Tris-HCl, pH 8.3, plus or minus 10 mM 2-mercaptoethanol or with 5 mM EDTA, pH 8.0.

2. Assay of stabilizing activity.

The stabilizing factor was routinely assayed using its stabilizing effect towards the thermal inactivation of ATP citrate lyase. ATP citrate lyase was passed on a column of Sephadex G-50 medium (1 x 9 cm) equilibrated with 10 mM Tris-HCl pH 7.4, containing 0.2 M KCl, 1 mM EDTA and 10 mM 2-mercaptoethanol. The protein concentration was adjusted to 1 mg/ml and 20 µl was mixed with 40 µl stabilizing factor and 10 µl 0.4 M Tris-HCl pH 8.4 was added to ensure that the pH of the mixture was 8.3. The mixture was incubated in a waterbath (Thermomix 1441, Braun Melsungen AG) set at 37° and samples were removed for assay of remaining activity of ATP citrate lyase at time intervals. The percent activity remaining after 30 minutes was calculated using linear regression analysis on semilogarithmic plots.

3. SDS polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis in the presence of SDS was performed according to Weber and Osborn (136) in 10% or 15% gels.

4. Amino acid analysis.

The determination of the amino acid composition is described in chapter II.

Automated Edman degradation was performed in a Beckman 890B Sequencer using the Polybrene/0.33 M Quadrol buffer procedure of Hunkapiller and Hood (137). Conversion of the thiazolinone derivatives to the corresponding phenylthiohydantoins was carried out by incubation with 1 M HCl at 80° for 10 minutes. For TLC, 0.2 mm thick silica gel plates with fluorescent indicator (E. Merck) were used and developed with 1,2-dichloroethane/acetic acid (10:1).

5. Protein determination using fluorescamine.

The concentration of the stabilizing factor, based on free amino groups, was determined by its reaction with fluorescamine, using GSH as a standard. The manual procedure of Böhlen *et al* (138) was followed except that 0.2 M sodium borate buffer pH 8.3 was used (0.2 M boric acid titrated to pH with NaOH). The fluorescence was read on a Turner Model 430 spectrofluorometer. The same buffer was used when

optimum pH for maximum fluorescence was determined. A standard GSH solution was made up to 1 mM in water. The actual concentration was determined by its extinction at 236 nm at pH 9.0 where $E(H_2O)=3162$ (145).

6. Carbohydrate determination.

The carbohydrate content was determined by the phenol-sulphuric acid method (139) using glucose as a standard. The colour was read at 490 nm after development.

7. Other procedures.

High voltage paper electrophoresis at pH 6.5 was performed in a Gilson High-Voltage Electrophorator (Model D) at 3000 Volt for 45 minutes as described by Ryle *et al* (140). The buffer system was pyridine-acetic acid-water (100:3:900 by volume).

Ascending paper chromatography was performed with Whatman No 4 paper and developed in n-butanol:pyridine:water (6:4:3 by volume).

Peptides were located with 1% ninhydrin in acetone (141) and carbohydrates with $AgNO_3$-NaOH spray (142).

C. RESULTS

1. Preparation of stabilizing factor in glucose medium.

The stabilizing factor was isolated from rat liver supernatant in the presence of 250 mM glucose. The slight modifications introduced did not change the properties of the final preparation. The elution profile of the factor on a Sephadex G-50 medium column is shown in Fig.17. This profile can be compared with the one obtained by Dunaway and Segal (83). Gel filtration can also be performed on a Sephadex G-25 fine column as shown in Fig.18. The use of fluorescamine to assay the eluant is described below.

To ascertain the purity of the factor, a sample was subjected to polyacrylamide gel electrophoresis in the presence of SDS. We could not see any protein band after staining with Coomassie blue, which is contrary to the finding of Dunaway and Segal(83). The factor preparation was subsequently subjected to high voltage paper electrophoresis at pH 6.5. Because of the similarity between the factor and glutathione noted by Dunaway and Segal (83), we ran commercial GSH as a standard together with other amino acids. After staining with ninhydrin, one major spot was found which migrated towards the anode almost identically to GSH (Fig.19). Careful inspection revealed another spot close to the origin which was very weak in intensity.

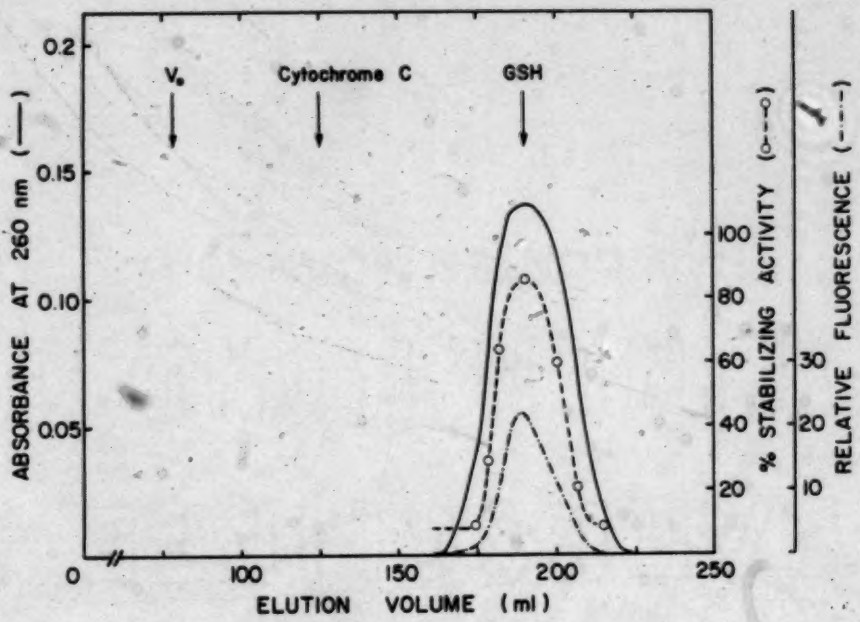

Fig.17. Chromatography of stabilizing factor on Sephadex G-50 medium (1.5 x 115 cm). The column was equilibrated and run at 6.5 ml/hr with 0.25 M glucose in 50 mM Tris-HCl buffer (pH 8.3). Absorbance at 260 nm, relative fluorescence (determined with the fluorescamine assay) and stabilizing activity were determined on the eluant. The column was calibrated with bovine serum albumin (for the void volume), cytochrome c and GSH.

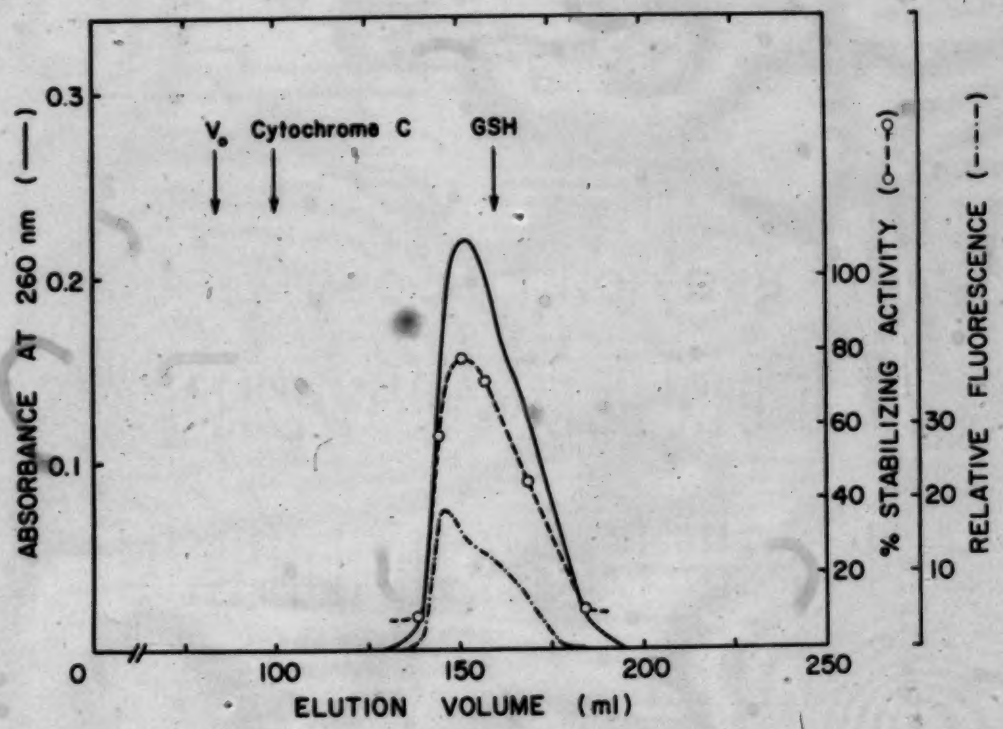

Fig. 18. Chromatography of stabilizing factor on Sephadex G-25 fine (1.5 x 107 cm). The column was equilibrated and run at 5.3 ml/hr with 0.25 M glucose in 5 mM EDTA (pH 8.0). Absorbance at 260 nm, relative fluorescence and stabilizing activity were determined on the eluant. The column was calibrated with bovine serum albumin (for the void volume), cytochrome c and GSH.

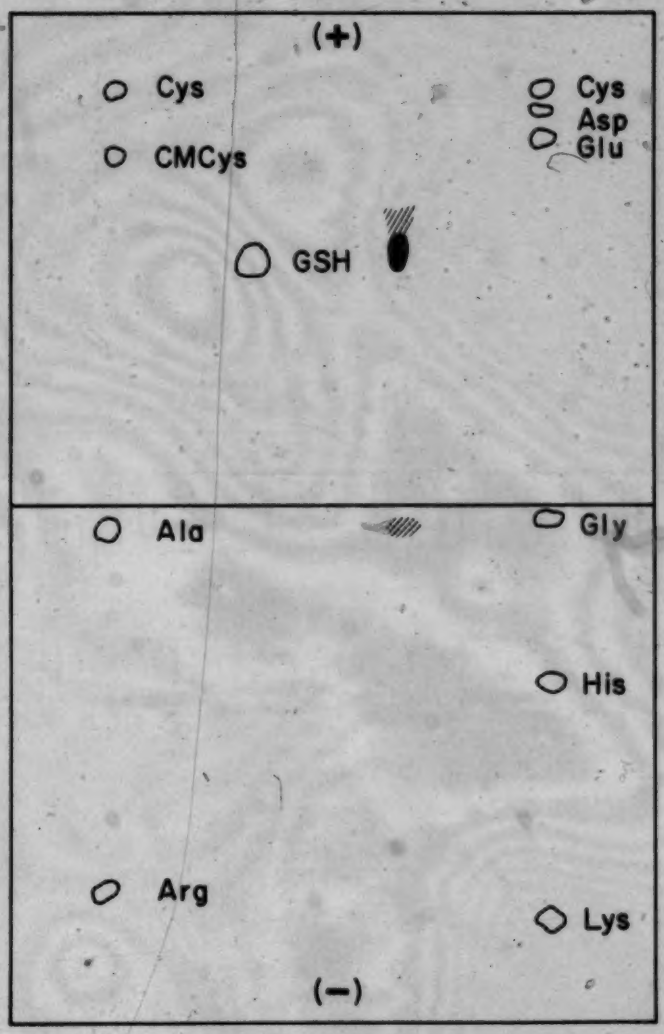

Fig.19. High voltage paper electrophoresis (pH 6.5) of the stabilizing factor. Factor was dialyzed against water and 40 nmoles of amine was applied to the paper. Electrophoresis was for 45 min. at 3,000 V.

2. Characterization of the stabilizing factor:

The amino acid composition of the factor was determined after acid hydrolysis with and without prior performic acid oxidation. The results are presented in Table VI. The oxidized sample gave an additional ninhydrin-positive peak eluting after 42 minutes in the initial buffer. We have not been able to identify this peak. Only traces (<5%) of other ninhydrin-positive material was present.

After dansylation and acid hydrolysis, a single spot corresponding to dansyl-glutamic acid was detected on a silica gel sheet.

When a sample was subjected to Edman degradation only the first step gave a UV-absorbing spot when analyzed by TLC. However, this spot did not comigrate with PTC-glutamic acid or any other known amino acid derivative. Both the amino acid composition and the presence of glutamate in the N-terminal position raised the possibility that the factor is glutathione or a glutathione polymer. GSH is a tripeptide, having the primary sequence H_2N-glu-cys-gly-COOH where the amino group of cys is coupled to the γ-carboxyl group of glu. The amino group of glu would react with PITC, but the γ-linkage would prevent cyclization and cleavage from taking place in the presence of anhydrous acid. Furthermore, if glutathione is present in the sample, it would be possible for the 1-chloro butane (used in the sequencer to extract the cleaved thiazolinones from the remaining peptide) to dissolve the PTC-glutathione; the spot

TABLE VI

Amino acid composition of the stabilizing factor after acid hydrolysis

Amino acid	Before performic acid treatment[1]	After performic acid treatment[1]
Glutamate	1.0	1.0
Glycine	1.11	1.62
Half-cystine	0.72	-
Cysteic acid[2]	-	1.12
X[3]	-	1.88

[1] All values are relative to glutamate.
[2] The colour factor for aspartic acid was used in calculating the amount of cysteic acid.
[3] X was eluted by the initial buffer (0.2 M sodium citrate, pH 3.25) with a retention time of 42 minutes. X has not been identified.

detected on the TLC plate could then be PTC-glutathione washed out of the sequencer reaction cup despite the presence of polybrene (which is used to reduce peptide extraction by 1-chloro butane). To test this idea, GSH was subjected to one Edman degradation cycle in the sequencer and the 1-chloro butane wash was done as before. The resultant extract was run on a TLC plate together with the first cycle product of the stabilizing factor and indeed they were found to comigrate. This suggests that the factor preparation contains glutathione.

3. Protein determination using fluorescamine.

During these studies it became evident that a fast and sensitive method of protein determination was needed. Neither the Lowry method (127), the microbiuret method (143) nor the dye binding method of Bradford (144) gave any colour reaction with either the stabilizing factor or with GSH. The latter method depends on the binding between protein and Coomassie blue and this could explain our earlier failure to detect the factor on SDS gel electrophoresis which was stained with Coomassie blue.

We were, however, successful with the fluorescamine assay as described by Böhlen *et al* (138). Fluorescamine reacts with primary amines to give a highly fluorescent derivative stable for a few hours in aqueous medium. The relative fluorescence is dependent on the pH (Table VII).

TABLE VII

Effect of pH on fluorescence of stabilizing factor, GSH and an amino acid mixture after reaction with fluorescamine

pH	Relative fluorescence		
	Amino acid mixture[1]	GSH	Stabilizing factor
8.0	1.0[2]	1.0	1.0
8.3	0.99	0.95	0.99
8.6	0.80	0.74	0.76
8.9	0.63	0.63	0.59
9.2	0.44	0.46	0.43
9.5	0.28	0.36	0.29

[1] The amino acid mixture contained glutamic acid, glycine and cysteine in equimolar ratio.
[2] The fluorescence at pH 8.0 for each mixture or substance was arbitrarily set to 1.0. It is not intended to imply equivalence of fluorescence at pH 8.0 for all three mixtures.

For all three mixtures the pH-optimum is between 8.0 and 8.5. Fig.20 shows a standard curve based on the reaction of GSH with fluorescamine at pH 8.3. The method is fast, sensitive and highly reproduceable, although we found that the resulting relative fluorescence was diminished with solutions of fluorescamine that were more than two weeks old. The fluorescamine solution was therefore renewed once a week.

4. The relationship between stabilizing factor and glutathione.

Amino acid analysis of the stabilizing factor suggests that it is similar to or identical with glutathione. However, the ultraviolet spectrum of the factor was found to be different from that of either reduced or oxidized glutathione, having a definite peak around 260 nm (Fig.21). Furthermore, while the spectrum of GSH is sensitive to pH (Fig.22), the spectrum of the stabilizing factor and of GSSG did not change appreciably over the pH range of 8.0 to 9.5. In addition, experiments reported in chapter VII show that the stabilizing factor does not contain any free sulfhydryl groups and furthermore, GSH cannot mimic the stabilizing effects of the factor in the concentration range where the latter is effective. Also, as documented in chapter VII, GSSG shows no stabilizing activity at all. Nevertheless considering the data it is possible that either glutathione

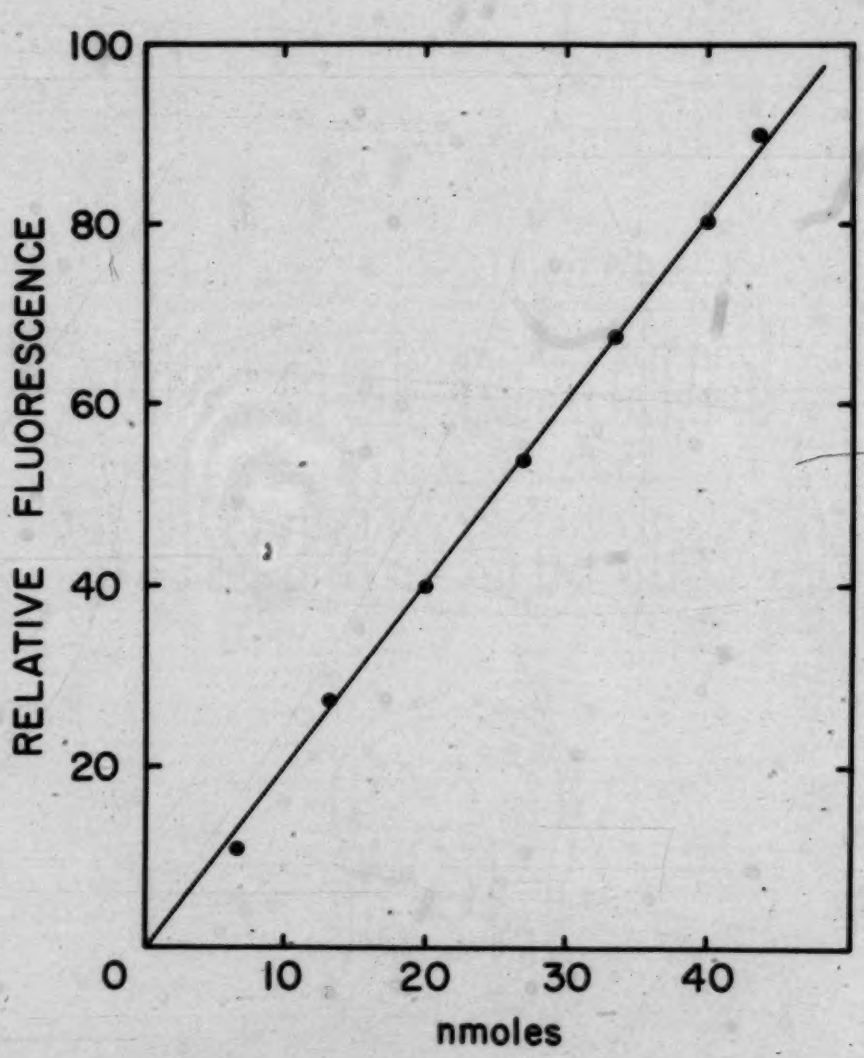

Fig.20. Correlation of relative fluorescence with the amount of GSH after reaction with fluorescamine.

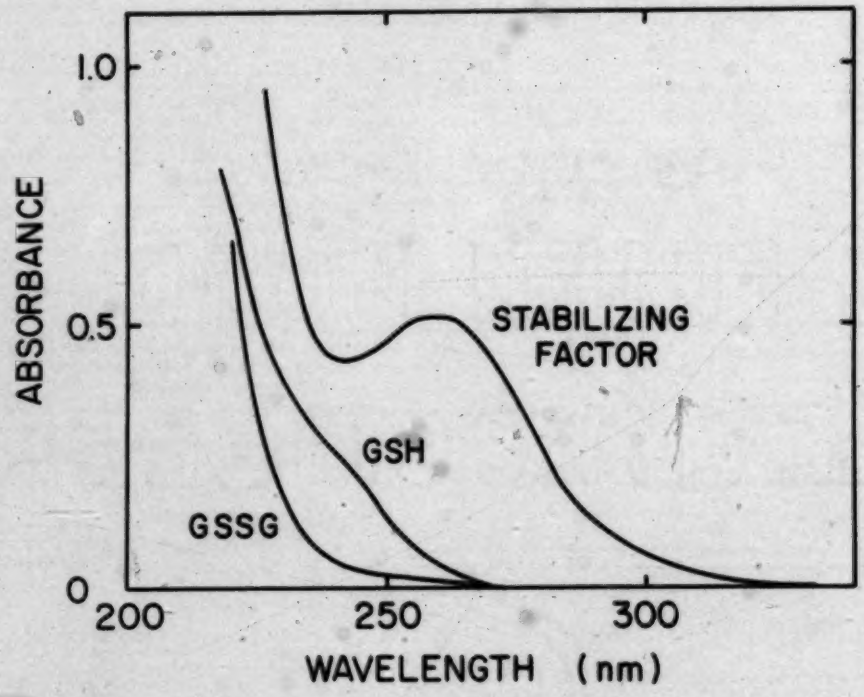

Fig.21. Ultraviolet spectra of the stabilizing factor, GSH and GSSG at pH 8.3. Reduced and oxidized glutathione were dissolved in 50 mM Tris-HCl (pH 8.3), to 0.2 mM and 1.6 mM final concentrations, respectively. The stabilizing factor in 50 mM Tris-HCl (pH 8.3) - 250 mM glucose was 0.5 mM in primary amine.

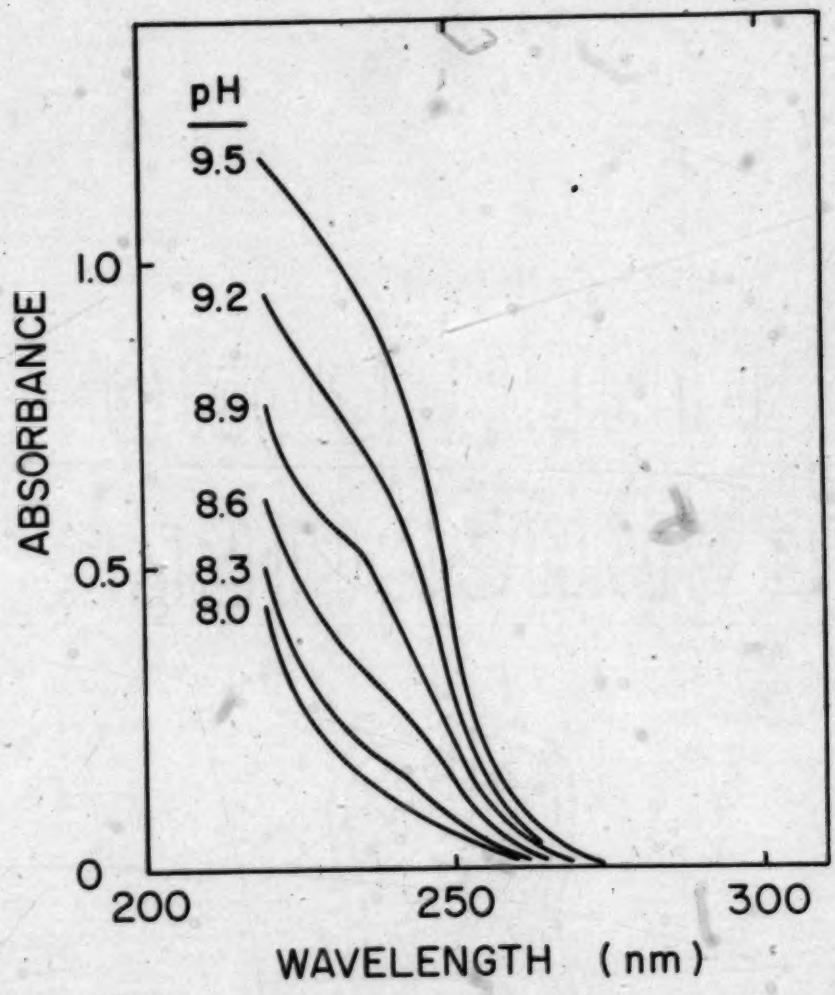

Fig.22. Effect of pH on the ultraviolet spectrum of GSH. Reduced glutathione was dissolved in 0.2 M sodium borate buffer at the indicated pH to a concentration of 0.2 mM.

copurifies with the stabilizing factor or that the factor is a glutathione derivative.

In our hands, GSH elutes close to the stabilizing factor both on Sephadex G-50 medium (Fig.17) and on Sephadex G-25 fine (Fig.18). This is in contrast to the results of Dunaway and Segal (83), who found that glutathione elutes after the factor on Sephadex G-50 chromatography. Furthermore, GSH has a molecular weight of approximately 300 and should be removed in the dialysis step. Indeed if freshly prepared GSH in 50 mM Tris-HCl buffer pH 8.3, containing 250 mM glucose is dialyzed 44 hours against the same buffer or buffer containing no glucose only about 10% remained in the dialysis bag (Table VIII), which agrees with the observations of Dunaway and Segal (83). If on the other hand GSH is stored at 0-5° for 6 days before dialysis, only 20-40% of the GSH was removed. The suspected aggregation phenomenon was verified when a sample of the same solution was passed through a Sephadex G-15 column, where the GSH was found in the void volume (Fig.23, bottom panel). The experiment was repeated, only this time a sample of the stored GSH was removed at different time intervals and passed through a Sephadex G-15 column equilibrated with 20 mM sodium phosphate buffer pH 8.0 (Fig.23, top three panels). From the elution behaviour, it appears that the apparent aggregate breaks down when diluted into buffer containing no glucose. The above glutathione solution was tested for stabilizing activity on ATP citrate lyase but none was found.

TABLE VIII

Recovery of GSH stored in glucose containing buffer, after dialysis

Days stored at 0-5° prior to dialysis	Dialyzed against	Recovery of GSH after dialysis (%)
0	Tris	7
0	Tris - glucose	13
2	Tris	33
2	Tris - glucose	39
6	Tris	63
6	Tris - glucose	76

A 1 mM solution of GSH was made up in 50 mM Tris-HCl pH 8.3, containing 250 mM glucose. After storage at 0-5° for the indicated number of days, it was dialyzed (Spectrapor 6 tubing) against 100 volumes of the same buffer or buffer devoid of glucose for 44 hours, with one change of buffer. The recovery of glutathione in the dialysis bag was estimated by the absorbance at 220 nm before and after dialysis.

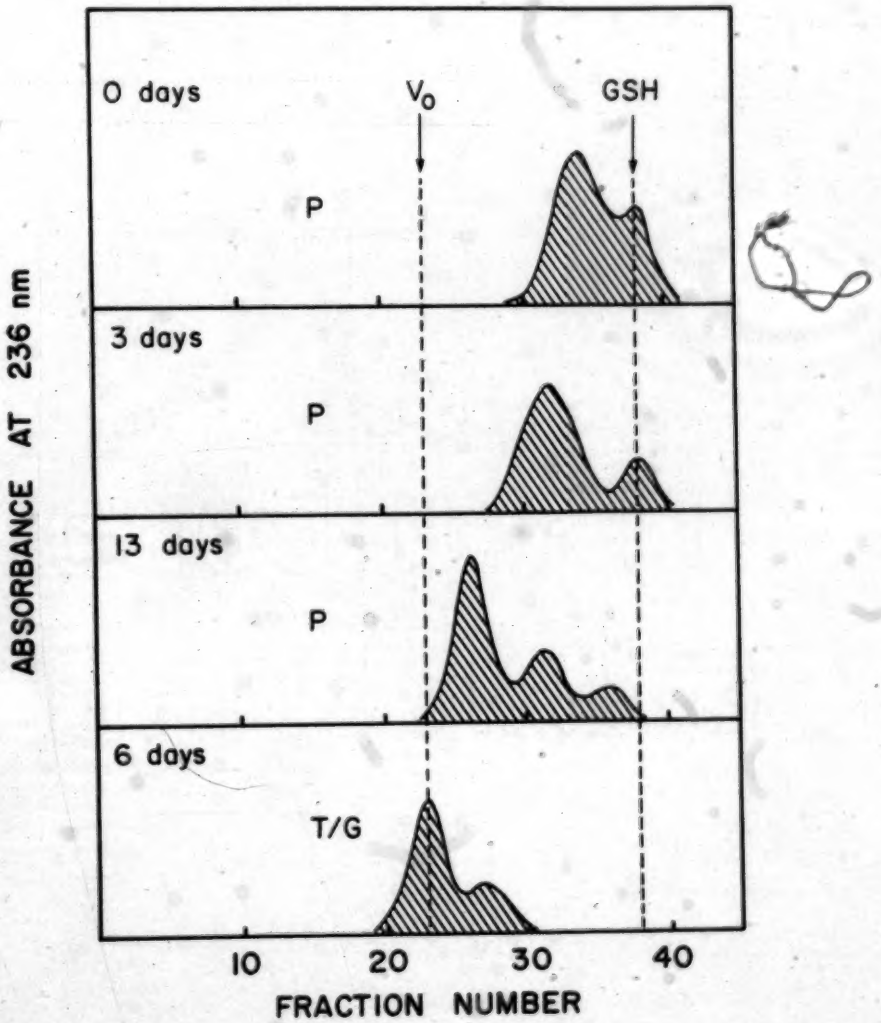

Fig.23. Chromatography of glutathione, stored in glucose-containing buffer on Sephadex G-15 column. A 1 mM GSH solution in 50 mM Tris-HCl (pH 8.3) - 250 mM glucose was stored at 0-5° the indicated number of days. A 2 ml sample was loaded on a Sephadex G-15 column (1 x 94 cm) equilibrated and washed at 2 ml/hr with either 20 mM sodium phosphate, pH 8.0 (=P) (the top three panels) or 50 mM Tris-HCl (pH 8.3) - 250 mM glucose (=T/G) (the bottom panel). The void volume was determined with soy bean trypsin inhibitor.

5. The stabilizing factor and glucose.

Dunaway and Segal (83) reported a molecular weight of the stabilizing factor of approximately 3,500. If the factor was a glutathione derivative behaving like GSH in its ability to complex in the presence of glucose to form a large aggregate, this could then break down in the absence of glucose. We therefore passed a sample of the factor through a Sephadex G-15 column equilibrated with phosphate buffer (Fig.24). When the eluant was tested, all the activity was recovered in an elution volume indicative of a mol.weight of 500 or less.

To eliminate the possibility of retardation on Sephadex due to its carbohydrate nature, the stabilizing factor was also run on a Biogel P2 column. The elution pattern (Fig.25) was essentially the same. Stabilizing activity could be superimposed on the fluorescamine peak; when the eluant was tested for the presence of carbohydrate this was detected eluting just after the stabilizing factor. Several other UV-absorbing peaks were observed on these columns, but none of them showed any stabilizing activity.

The above experiments suggest that the stabilizing factor aggregates in the presence of glucose and more important that the factor is still active as a small molecule. How then would the factor behave if isolated from rat liver in buffer totally devoid of glucose? In order to answer this question, the stabilizing factor was prepared from rat liver in 20 mM sodium phosphate buffer pH 8.0 and

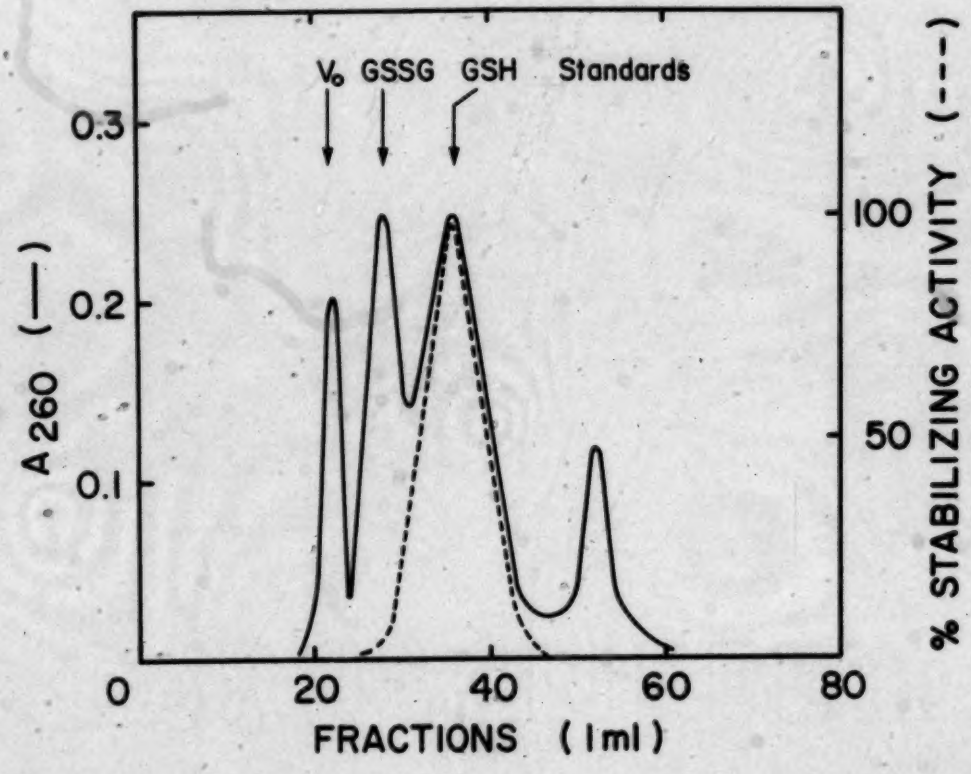

Fig.24. Chromatogram of stabilizing factor on Sephadex G-15 column. Stabilizing factor in 50 mM Tris-HCl (pH 8.3) - 250 mM glucose was chromatographed on Sephadex G-15 (1 x 94 cm) equilibrated and run at 2 ml/hr with 20 mM sodium phosphate buffer (pH 8.0). Absorbance at 260 nm and stabilizing activity was determined on the eluant. The column was calibrated with soy bean trypsin inhibitor (for the void volume), GSSG and GSH.

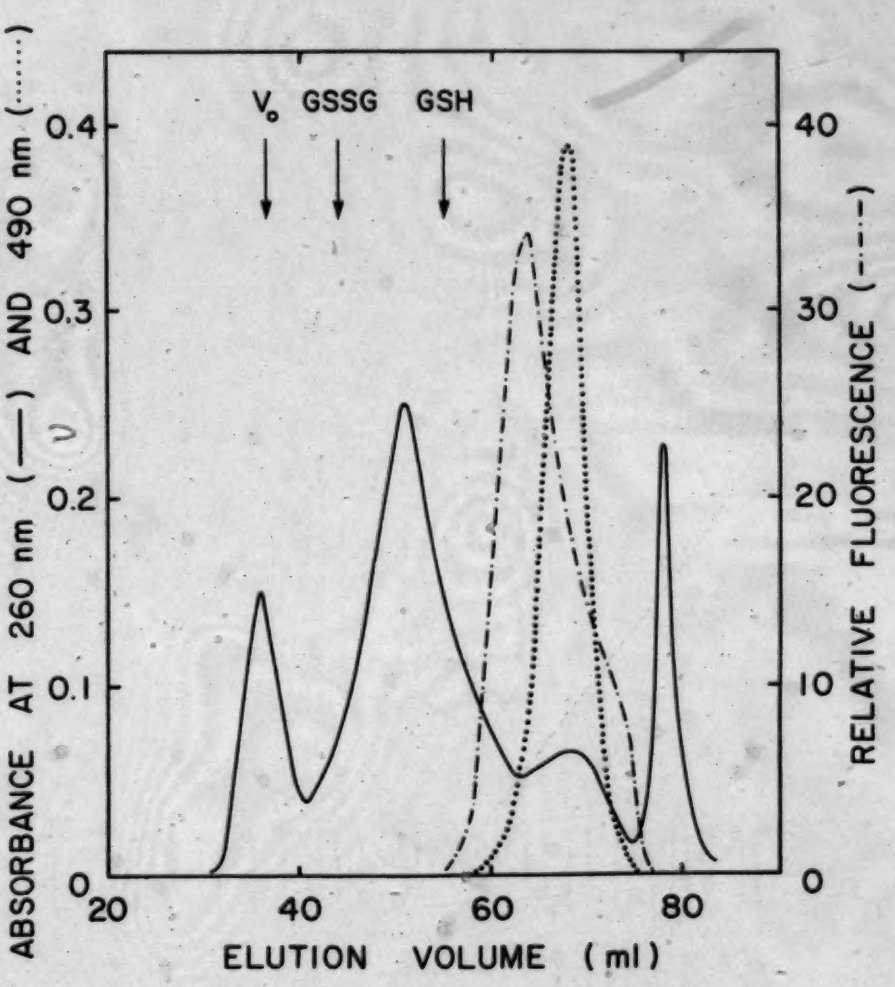

Fig.25. Chromatogram of stabilizing factor on a Biogel P2 column. Stabilizing factor in 50 mM Tris-HCl (pH 8.3) - 250 mM glucose was chromatographed on a Biogel P2 column (1 x 94 cm) equilibrated and run at 2 ml/hr with 20 mM sodium phosphate buffer (pH 8.0). Absorbance at 260 nm, stabilizing activity and carbohydrates (abs. at 490 nm) were determined on the eluant. The column was calibrated with soy bean trypsin inhibitor (for the void volume), GSSG and GSH.

purified as before. After dialysis the solution was concentrated over a UM2 membrane. We were unable to detect stabilizing activity in the retentate but we found activity in the filtrate, indicative of a mol.weight of 1,000 or less.

6. Analysis of small mol.weight stabilizing factor.

The stabilizing factor recovered after gelfiltration on Sephadex G-15 (Fig.24) or on Biogel P2 (Fig.25) was analyzed by high voltage paper electrophoresis at pH 6.5. A total of six ninhydrin-positive spots were detected, one neutral, three acidic and two basic (Fig.26). We have not been able to identify any of these spots as the stabilizing factor either individually or in combination. To determine whether the electrophoresis procedure inactivated the factor, an experiment was performed where equal amounts of factor was applied on two papers. One paper was then run at 3,000 Volt and for 20 minutes instead of the usual 45 minutes. The other paper was merely wetted with the electrophoresis buffer and allowed to dry. Both papers were then eluted with water overnight and the eluant was concentrated back to its original volume by lyophilization. All of the activity was recovered from the paper which had not been electrophoresed, while none was found in the eluant of the other paper.

The stabilizing factor pooled after Sephadex chromatography was analyzed for its carbohydrate content,

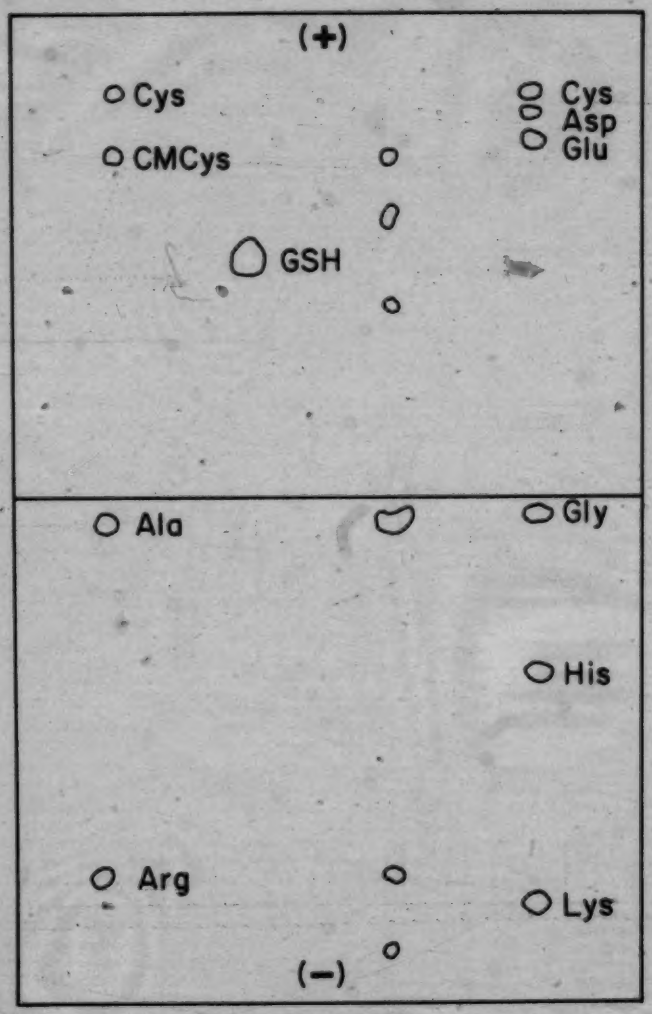

Fig.26. High voltage paper electrophoresis (pH 6:5) of stabilizing factor after gel filtration on a Biogel P2 column. 160 nmoles of stabilizing factor was applied and electrophoresis was performed at 3,000 V for 45 min.

quantitatively with the phenol-sulphuric acid method using glucose as a standard and qualitatively by paper chromatography. The factor, (0.3 mM in amine concentration) had a carbohydrate concentration equivalent to 0.25 M glucose. Two spots were visualized by spraying for carbohydrate (Fig.27), one heavily stained (R_f= 0.94 relative to glucose) referred to as the C-component and one slightly coloured spot moving just off the origin. This latter spot was not homogeneously coloured, but rather uneven such that the part farthest away from the origin was more intensily coloured than the rest. Furthermore, when the paper was sprayed with ninhydrin only the slower moving spot could be visualized, which will be referred to as the P-component. Preparative paper chromatography was performed and both spots were eluted with buffer and brought back to the original volume. All of the activity was recovered in the P-component. Ultraviolet spectra were taken of both the P- and C-component (Fig.28). Both components showed a maximum around 250-260 nm, although the one of the P-component was more well defined with a characteristic pattern.

In order to achieve large-scale separation of the carbohydrate from the protein, the stabilizing factor solution was passed repeatedly through a Biogel P2 column and each time only tubes in the beginning of the activity peak were pooled. A factor solution so prepared was reduced in its carbohydrate content to 0.6 moles of glucose to 1 mole of amine. However, high voltage paper electrophoresis

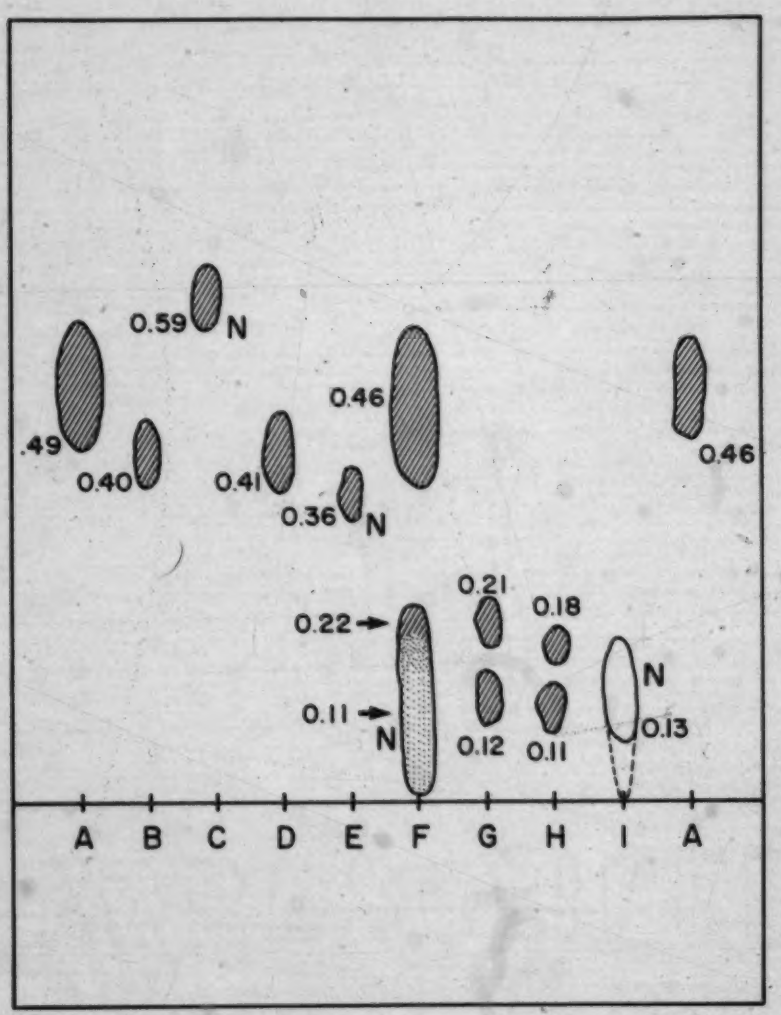

Fig.27. Paper chromatogram of the stabilizing factor. Stabilizing factor (230 µg carbohydrate) was applied on a Whatman paper No 4 together with 20 µg of each of the standards. A) glucose, B) glucosamine, C) N-acetylglucosamine, D) galactose, E) galactosamine, F) stabilizing factor, G) glucuronic acid, H) galacturonic acid, and I) glutathione. Shaded areas are carbohydrate and N denotes ninhydrin-stained spot. The numbers are the R_f of respective spot (measured to the center of the spot) relative to solvent (n-butanol:pyridine:water; 6:4:3, volume by volume).

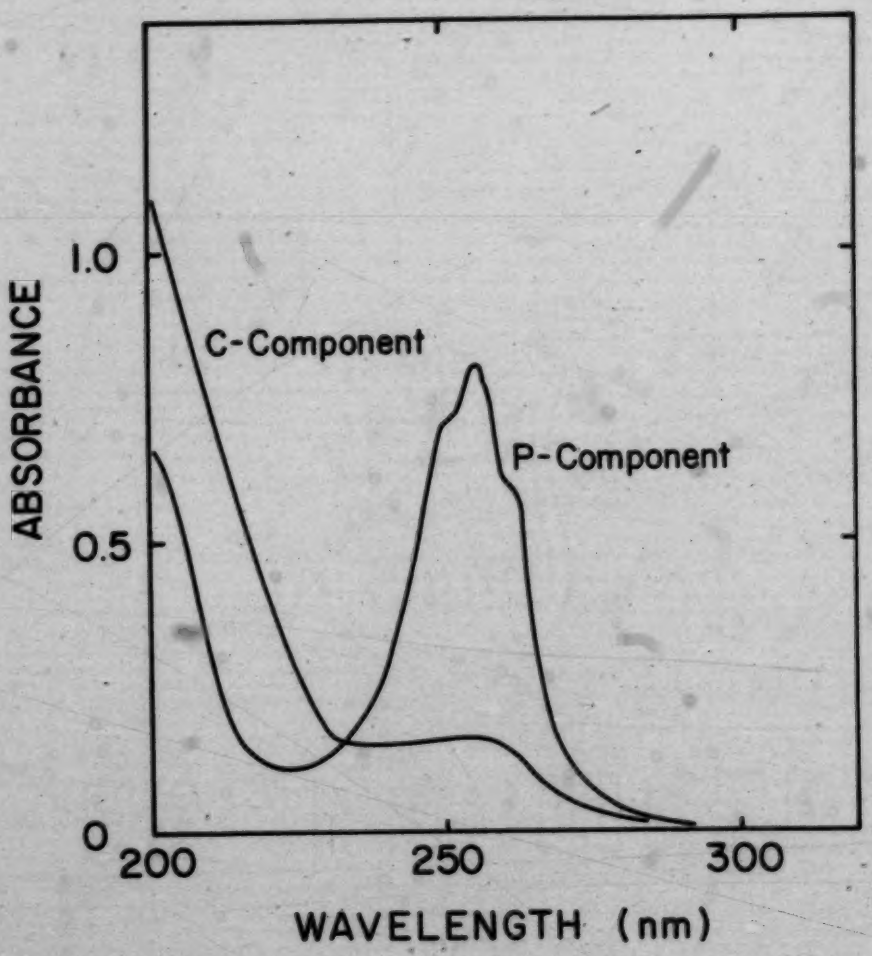

Fig.28. Ultraviolet spectra of the components of the stabilizing factor at pH 8.0 (20 mM sodium phosphate buffer) after preparative paper chromatography. The P-component was 0.2 mM in amine and the C-component was 5 mM in glucose.

patterns of this solution were not different from before (see Fig.26).

D. DISCUSSION

Dunaway and Segal (83) found that the stabilizing factor lost its activity during storage, but could be stabilized by the addition of glucose. Accordingly they isolated, purified and characterized the factor in buffer containing 250 mM glucose. We followed their procedure in this respect, and when characterizing the factor we also found strong indications of the presence of glutathione or a derivative thereof. The amino acid composition, the determination of glutamic acid as the N-terminal residue and its resistance to standard sequencing methods, where the resultant PTH-derivative comigrated with standard PTH-GSH on TLC plates, all suggest that the factor preparation after the Sephadex G-50 filtration step contains GSH or a glutathione-like molecule.

Could glutathione play some role in regulating enzyme turnover? Glutathione is the most abundant low molecular weight thiol in the cell. While the reduced form (GSH) is present in relatively high concentrations (1-10 mM), the concentration of oxidized glutathione (GSSG) is only about 1% of the reduced form (147). The ratio is maintained by the highly active glutathione reductase, which requires NADPH as a cofactor (148). The NADPH is supplied by glucose-6-phosphate dehydrogenase and indeed in red blood cells the activity of the pentose phosphate pathway is correlated with the GSSG/GSH ratio (149).

A small rise in the concentration of GSSG has been

shown to inhibit protein synthesis in lysates of rabbit red blood cells (151). In fact using diamide, a specific thiol oxidizing agent, Kosower and Kosower (152) have shown that a variety of metabolic processes are strongly affected by disturbances of the thiol-disulfide status of the cell.

GSH is involved in a variety of biochemical reactions; for instance, it is an important reagent in the detoxification of foreign compounds (153) and it has been shown to participate in amino acid transport (154), where the γ-glutamyl group of GSH is coupled to the amino acid being transported across the membrane. This process is catalyzed by a γ-glutamyl transpeptidase and the GSH used up in the reaction is regenerated in the γ-glutamyl cycle. The enzymes of this cycle are clearly involved in the turnover of GSH, and the interest in this cycle increased dramatically by the recent finding that these enzymes are induced in parallel with the accumulation of abnormal proteins in chicken embryo cells (155).

A large percentage of the total GSH may exist bound to proteins as mixed disulfides (156). Formation of mixed disulfides might play an important role in assuring the correct formation of proteins during synthesis, in preventing proteins from denaturation and in modulating membrane activities, such as regulation of insulin mediated glucose transport (34). Changes in the activity of a few enzymes of the carbohydrate metabolism could be attributed to the formation of mixed disulfides with low molecular weight

thiol compounds. Liver fructose-1,6-diphosphatase has been shown to be activated by mixed disulfide formation, homocystine being the best activator (157). This enzyme was inactivated by GSH. Inactivation of rat liver glycogen synthetase D by GSSG was demonstrated to be the result of mixed disulfide formation between GSSG and the sulfhydryl groups of the enzyme. Reactivation could be accomplished by GSH in the presence of glucose-6-phosphate (158). Also a factor that inactivated phosphorylase phosphatase was isolated from rabbit liver and identified as GSSG (159).

It is evident that glutathione is involved in many deversified biological processes, many of them poorly understood. It is therefore conceivable that glutathione also plays some role in the regulation of protein turnover. In discussing the purification and the characterization of the stabilizing factor and its relationship to glutathione, two possible explanations for the experimental results must be considered. Firstly, the possibility that GSH copurifies with the factor, and secondly, that the factor is a glutathione derivative or that the factor is simply coupled to GSH.

Dunaway and Segal (83)interpreted the absence of UV-absorbing peaks after the elution of the factor of Sephadex gel filtration as a sign that GSH was effectively removed in the dialysis step. In addition, they found that standard GSH was separated from the factor when these were chromatographed together. In contrast we were not able to

distinguish the elution volume for the stabilizing factor from that for GSH on gel filtration (Fig.17 and 18). Investigation of the behaviour of GSH in the dialysis step revealed an interesting apparent aggregation in glucose buffer (Table VIII), which was verified upon Sephadex G-15 filtration (Fig.23). Retainment of intracellular GSH upon dialysis would result in copurification of the stabilizing factor with GSH and in the gel filtration step both of them would elute together. If GSH were present in our factor preparation as an impurity its concentration compared to the factor could be very much higher, because the intracellular concentration is high as discussed above. From this it follows that any characterization of the factor would yield a picture resembling GSH.

Gel filtration of the stabilizing factor on Sephadex G-15 (Fig.24) or on Biogel P2 (Fig.25) in the absence of glucose shows a much smaller molecular weight than originally estimated by Dunaway and Segal (83). Glucose might cause an aggregation of the factor, but the activity does not seem to depend on the aggregation state. We have tried without success to reaggregate the factor by incubating the small mol.weight factor in fresh glucose buffer. It appears that aggregated factor is an artifact caused by the presence of glucose during the purification, since isolation in the absence of glucose yields a small mol.weight factor that penetrates the UM2 membrane (1,000 mol.weight cut off), although breakdown of an aggregate might have happened

during the first purification steps.

Most, but not all, of the carbohydrate can be removed by repeated gel filtration and discriminate pooling. Also paper chromatography separates the activity from most of the carbohydrate (Fig.27). The identity and role of the remaining carbohydrate moiety remains to be determined. It might be important for activity, and the stabilizing factor could be a small glycosylated derivative of GSH (146). The stabilizing factor, earlier thought to be pure after Sephadex G-50 filtration, resolves into 6 ninhydrin-positive spots after Biogel P2 upon application to high voltage paper electrophoresis at pH 6.5 (Fig.26). This is puzzling, as is the fact that we are not able to regain the activity after electrophoresis and elution of the paper. The possibility that the factor or part of the factor migrates into the electrophoresis buffer must be considered.

The remaining question is whether the factor is identical to glutathione. This does not seem to be the case, since Dunaway and Segal (83) found no stabilizing activity with either reduced or oxidized glutathione. We have come to similar conclusions, based on experiments reported in chapter VII. Furthermore, the factor shows a very characteristic UV-spectrum which is different from either GSH or GSSG (Fig.21). The absorption maximum near 260 nm was not seen by Dunaway and Segal (83), but it is possible that these investigators measured the UV-spectrum of a very dilute sample, in case the peak at 260 nm could be missed. In fact

at very high concentration we observed another plateau at about 310 to 320 nm.

Glutathione is a strong nucleophilic compound, and it is therefore important, when evaluating the GSH-GSSG status of the cell, to consider various hidden forms, such as mixed disulfides, thiolesters (160) and other glutathione derivatives (161,162). The roles of these various forms are not well understood. The possibility that the stabilizing factor is a new hidden form of GSH, cannot be excluded, although this remains to be shown. Attempts to characterize the factor with respect to its identity and mode of action are described in chapter VII.

VII. MODE OF ACTION OF THE STABILIZING FACTOR

A. INTRODUCTION

The two lipogenic enzymes phosphofructokinase and ATP citrate lyase are affected in a similar way by a factor obtained from rat liver supernatant. The factor stabilizes these two enzymes *in vitro* against thermal and lysosomal inactivation (83,chapter V of this work). The interest in the stabilizing factor as a possible physiological regulator of protein turnover stems largely from the observation that the level of the factor in rat liver can be altered by dietary and hormonal manipulation. The level of the stabilizing factor is altered in the same direction as the level of the lipogenic enzymes, but precedes the enzymes in time upon refeeding a high carbohydrate diet to starved rats (83) or upon insulin treatment of diabetic rats (128).

Dunaway and Segal (83) purified the stabilizing factor to apparent homogeneity, and reported that its molecular weight is approximately 3,500 and that it is heat labile, susceptible to pronase digestion and, as discussed earlier, they suggested that its chemical structure might be related to glutathione.

We have attempted to purify and characterize the stabilizing factor (chapter VI) in order to confirm the results obtained by Dunaway and Segal (83) and to further increase our understanding of the possible means of interaction between the factor and its target enzyme. In

this chapter, I describe studies of the the mechanism of the thermal inactivation of ATP citrate lyase and of the nature of the stabilizing effect of the factor on the enzyme. In addition a variety of compounds have been tested either for stabilizing activity or for ability to chemically modify the factor in an indirect attempt to identify the stabilizing factor.

B. METHODS

1. Determination of stabilizing activity and protein concentration.

The procedure for thermal inactivation of ATP citrate lyase and quantitation of the stabilizing factor and GSH is described in chapter VI.

2. Iodoacetamide treatment of ATP citrate lyase.

ATP citrate lyase (1 mg/ml) was treated with iodoacetamide as follows. 20 µl enzyme was mixed with 40 µl 20 mM sodium phosphate pH 8.0 or stabilizing factor (4.4 mM) and 10 µl iodoacetamide in 0.5 M sodium phosphate pH 8.0 was added to produce the desired final concentration of 0.1 mM, 1 mM and 10 mM. The mixture was incubated at 25° in a waterbath and samples were removed at time intervals for assay of remaining activity.

3. Quantitation of sulfhydryl groups.

Estimation of free sulfhydryl groups was done by using DTNB as described by Ellman (163). Determination of monothiols after prior reduction of sulfide bridges was done according to the method developed by Zahler and Cleland (164).

4. Iodoacetamide treatment of the stabilizing factor.

Stabilizing factor was treated with 1 mM iodoacetamide at pH 8.0 for 4 hours at 25°. Cysteine was then added to 1.2 mM final concentration and the mixture was incubated at 37° for 90 minutes, a procedure which removes the unreacted iodoacetamide. The stabilizing factor was then tested for activity and compared with a control, where iodoacetamide was allowed to react with cysteine before the factor was added.

5. Hydroxylamine treatment of the stabilizing factor.

A 4 M hydroxylamine solution was neutralized with an equal volume of a 4.6 M KOH just prior to use (165) and a suitable volume was added to a solution of stabilizing factor (4.4 mM) to produce 0.2 M final concentration. A sample of the solution was tested for activity at zero time and after 4 hours incubation at 25°.

6. Proteolytic digestion of the stabilizing factor.

The stabilizing factor was treated with CMC-pronase at 25° according to Dunaway and Segal (83). To 1 ml samples of solution of stabilizing factor, obtained either after Sephadex G-50 filtration in the presence of glucose or after Sephadex G-15 filtration in the absence of glucose, (30.6 mM and 4.4 mM in the concentration of primary amines,

respectively) were each treated with 10 mg CMC-pronase (dry weight) for 90 minutes. Assay of stabilizing activity was performed in the range of factor concentration where the standard curve is linear.

7. Determination of CoA.

The assay of CoA using the coupled enzymatic assay of ATP citrate lyase was performed essentially as described by Srere (166). Each cuvette contained 200 mM Tris-HCl pH 8.4, 20 mM potassium citrate, 10 mM $MgCl_2$, 5 mM DTT, 2 units malate dehydrogenase per ml, 0.14 mM NADH, 5.25 mM ATP, 8 µg ATP citrate lyase (specific activity = 2.2 units/mg) and 0.1 - 0.75 µM CoA. The reaction was initiated by the addition of enzyme and the reaction allowed to proceed until all the CoA was exhausted. The amount of NADH oxidized, measured as the difference in absorption at 340 nm before and after the reaction, was plotted against the amount of CoA added.

8. Determination of citrate.

An assay for citrate, equivalent in principle to that of CoA, was developed. 0.25 M KCl was included in the assay medium (124), the concentration of CoA was 0.1 mM and that of potassium citrate was varied between 1 and 5 µM.

9. Preparation of Glucose Tolerance Factor.

The Glucose Tolerance Factor was prepared as a chromium complex as described by Toepfer *et al* (167). The product was identified by its ultraviolet spectrum (167).

C. RESULTS

1. Mode of action of the stabilizing factor.

It is known that ATP citrate lyase is very susceptible to oxidation with accompanying loss of most of its activity (131). Furthermore, stored enzyme which had lost activity can be reactivated by incubation with DTT. Based on the pH-dependence of the thermal inactivation of ATP citrate lyase (chapter V), we suspected that this process might involve oxidation of especially vulnerable sulfhydryl groups on the enzyme which are important for activity. It was therefore of interest to test the effect of various thiol-containing reagents as possible protectors against thermal inactivation of ATP citrate lyase. While 2-mercapto-ethanol at 10 mM final concentration showed little stabilizing effect (Table IX), 1 mM DTT fully protected the enzyme and 10 mM GSH showed intermediate protection.

All experiments described in chapter V that demonstrated the effect of the stabilizing factor were performed in the presence of 2-mercaptoethanol. In order to differentiate between the effect of the factor and that of the reducing reagent, the effect of the factor was studied in the absence of 2-mercaptoethanol. Under these conditions the stabilizing factor was very ineffective as a protector of ATP citrate lyase (Fig.29). The reason for this is not clear, but we have, in all subsequent experiments, tested for stabilizing activity in the presence of 2.8 mM 2-mercaptoethanol.

TABLE IX

Effect of reducing reagents on thermal inactivation of ATP citrate lyase[1]

Addition	Activity remaining after 30 min. (%)
None	32.5 ± 5.0 (4)[2]
10 mM 2-mercaptoethanol	43.0 ± 6.4 (4)
10 mM GSH	69.4 ± 2.2 (3)
1 mM DTT	105.6 (1)

[1] 100 µl ATP citratelyase (1 mg/ml) was mixed with 50 µl 50 mM Tris-HCl (pH 8.3) - 250 mM glucose, 15 µl 50 mM Tris-HCl (pH 8.3) with or without reducing reagent and 10 µl 0.4 M Tris-HCl, pH 8.3 and the mixture was incubated at 37°. At time intervals samples were removed for assay of ATP citrate lyase. The percent activity remaining after 30 min. was calculated using linear regression analysis on semilogarithmic plot.
[2] Number of experiment.

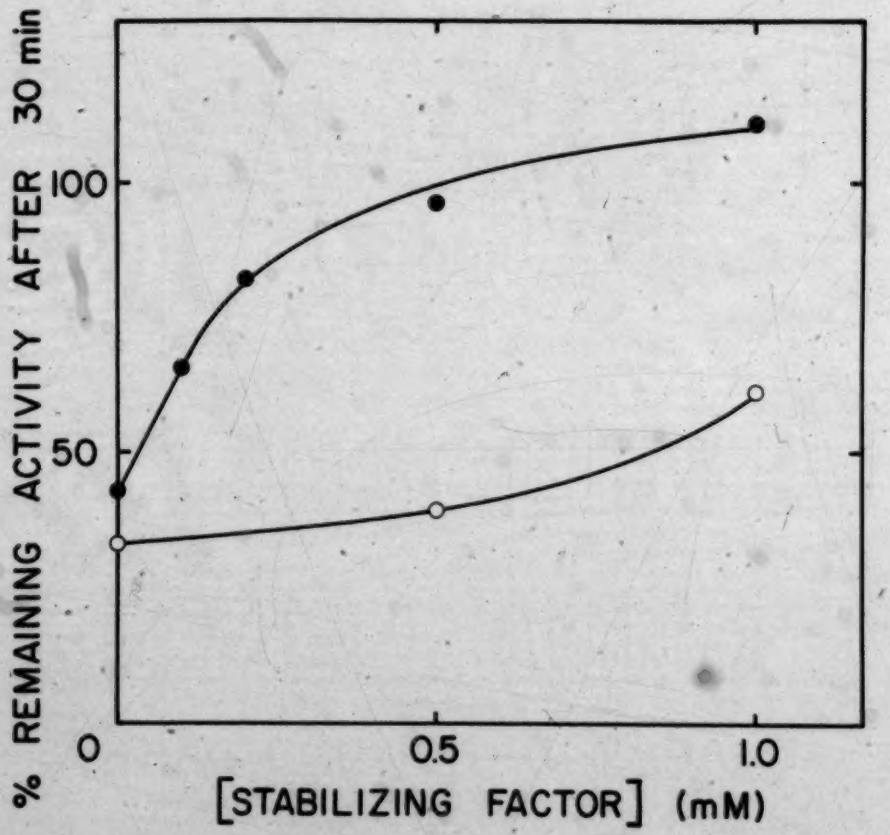

Fig.29. The effect of stabilizing factor concentration on thermal inactivation of ATP citrate lyase in the presence or absence of 2-mercaptoethanol. 100 µl ATP citrate lyase (1 mg/ml) was incubated with various amounts of stabilizing factor in 50 µl 50 mM Tris-HCl (pH 8.3) - 250 mM glucose. 15 µl 50 mM Tris-HCl (pH 8.3) plus (●) or minus (○) 2-mercaptoethanol and 10 µl 0.4 M Tris-HCl (pH 8.3) were added and the mixture was incubated at 37°. Samples were removed at time intervals for assay of ATP citrate lyase. The percent activity remaining after 30 min. (triplicate incubation mixtures) was calculated using linear regression analysis on semilogarithmic plot.

The dependence of the stabilizing effect on factor concentration (measured as primary amines using GSH as a standard) is shown in Fig.30. The stabilizing activity increased sharply reaching a maximum at about 1 mM and then declined. The effect of GSH was tested under identical conditions. GSH showed little effect in the concentration range where the factor was most effective, but could induce thermal stability of ATP citrate lyase at higher concentrations.

The concentration dependence of the effect of the stabilizing factor is very distinctive. A similar decline at high concentrations of the factor was reported by Dunaway and Segal (83). They found that the decrease in activity at excess factor concentrations could be eliminated by including 10 mM GSH in their incubation mixture. Accordingly, we repeated the experiment and included GSH in the mixture at a final concentration of 10 mM. Instead of an elimination of the decrease in activity, we observed an additive effect of GSH (Fig.31). The difference in the behaviour of ATP citrate lyase to that of phosphofructokinase was further reflected by the inability of DTT and GSH alone to confer thermal stability to the latter enzyme (83).

These experiments suggest an interrelationship between thermal stability and the oxidation state of ATP citrate lyase. It should be noted that the ATP citrate lyase preparation used in these experiments was not fully active, and that attempts to produce a fully active reduced enzyme

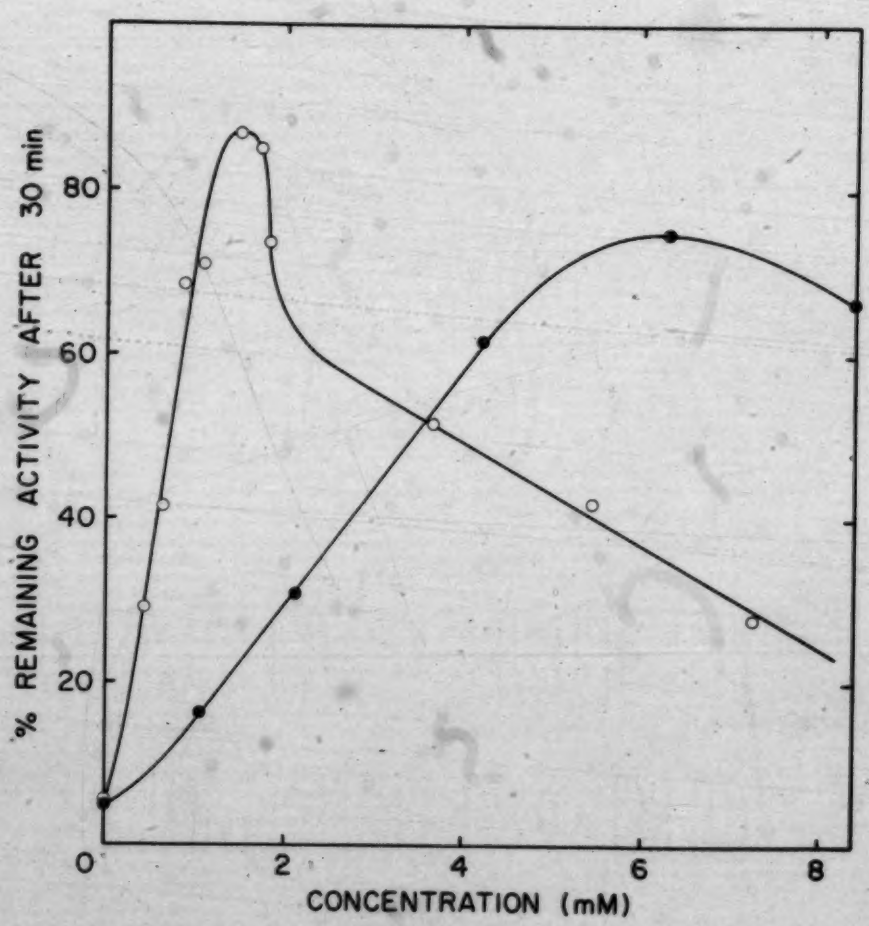

Fig.30. Effect of stabilizing factor concentration and GSH concentration on thermal inactivation of ATP citrate lyase. 20 µg enzyme was incubated with various amounts of stabilizing factor (○) or GSH (●) in a final volume of 70 µl at 37° and pH 8.3. Samples were removed at time intervals for assay of ATP citrate lyase. The percent activity remaining after 30 min., calculated using linear regression analysis on semilogarithmic plot, was plotted against final concentration of stabilizing factor and GSH. Each point represents a single incubation mixture.

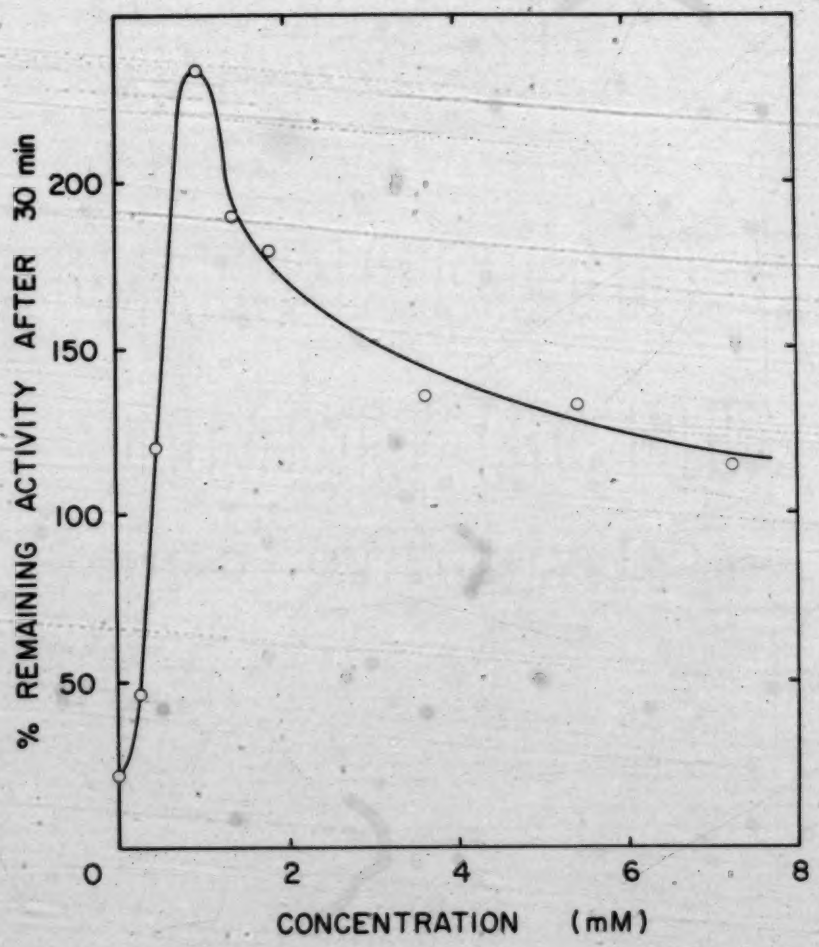

Fig.31. Effect of stabilizing factor concentration on thermal inactivation of ATP citrate lyase in the presence of GSH. 20 µg enzyme was incubated with various amounts of stabilizing factor in the presence of 10 mM GSH at 37° and pH 8.3 in a final volume of 70 µl. Samples were removed for assay of ATP citrate lyase. The percent activity remaining after 30 min., calculated using linear regression analysis on semilogarithmic plot, was plotted against final concentration of stabilizing factor. Each point represents a single incubation mixture.

by incubating with 10 mM DTT, followed by removal of DTT on a short Sephadex column have failed, since removal of reducing reagent leads to a rapid loss of activity. If the enzyme is thermally inactivated, however, activity can be restored by adding DTT in excess of 5 mM. Addition of stabilizing factor, on the other hand, fails to restore the activity. It is possible then, that the stabilizing factor is able to protect certain vulnerable sulfhydryl groups from oxidation, but once they have been oxidized, the factor cannot of itself cause reduction and accompanying reactivation. This hypothesis was tested in an experiment shown in Fig.32. The factor was added to the incubation mixture either initially or after thermal inactivation of ATP citrate lyase had proceeded for a certain period of time. When the factor was added, the rate of inactivation decreased appreciably and the new rate obtained was similar in all cases. When 2 mM DTT replaced the factor, the activity increased to the original level or above when it was added at zero time and after 3 minutes of inactivation, but not after 10 minutes. Earlier observations have shown that 5 mM or more of DTT is needed to restore the activity after extensive inactivation and if the enzyme is left in an inactivated state for several hours 10 mM DTT only partially restores the activity.

Cottam and Srere (131) have shown that ATP citrate lyase is very sensitive to inhibition by DTNB. ATP citrate lyase is also inhibited by iodoacetamide in a time- and

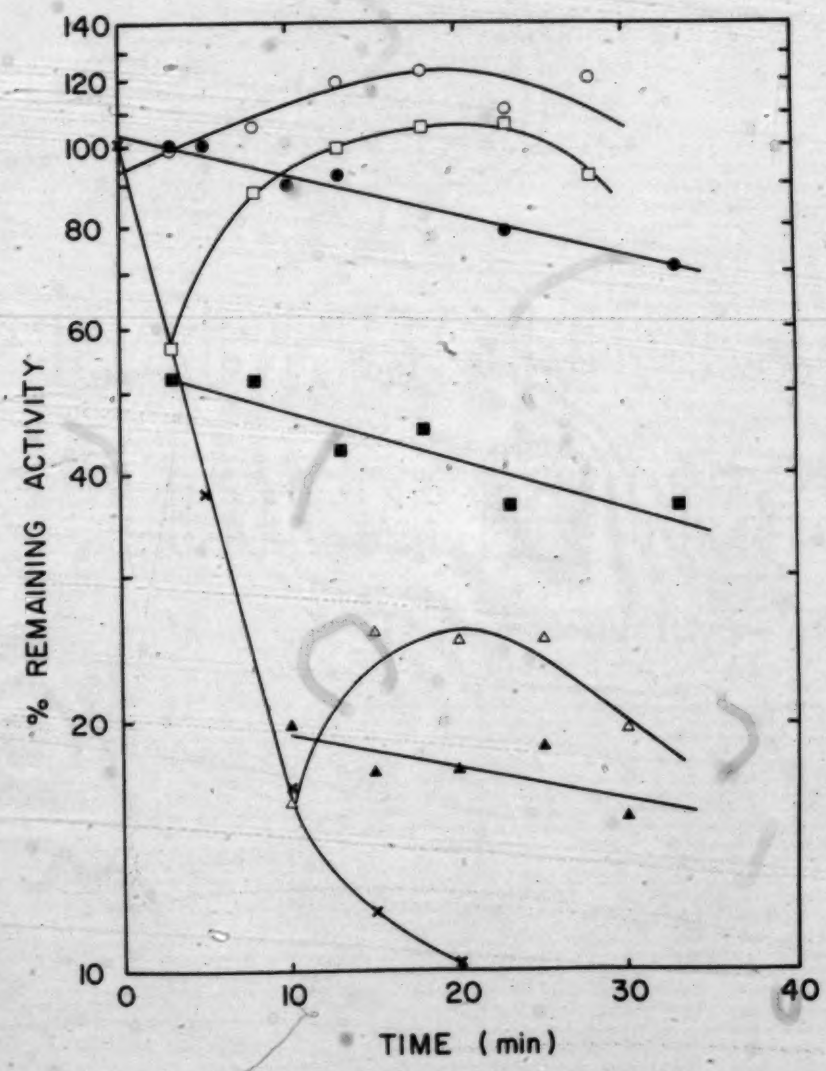

Fig.32. Effect of stabilizing factor or DTT addition during thermal inactivation of ATP citrate lyase. 20 µl enzyme (1 mg/ml) was mixed with 30 µl 20 mM sodium phosphate (pH 8.0) and 10 µl 0.4 M Tris-HCl (pH 8.4) and incubated at 37°. Samples were removed at time interval for assay of ATP citrate lyase (x). At start of the incubation (○●), after 3 min. (□■) and after 10 min. (△▲) 10 µl stabilizing factor or DTT (in 20 mM sodium phosphate, pH 8.0) were added to final concentration of 2.5 mM and 2.0 mM, respectively. Incubation was continued and samples were removed at time intervals for determination of enzyme activity. Solid symbols represents the addition of stabilizing factor and open symbols that of DTT.

concentration-dependent manner (Fig.33). With 10 mM iodoacetamide half of the activity is lost in less than 5 minutes. In Fig.34 it is shown that this inactivation by iodoacetamide can be prevented by the addition of the stabilizing factor. This protection lends further support to the idea that the stabilizing factor is interacting with the sensitive sulfhydryl groups of ATP citrate lyase.

2. Properties and stabilities of the stabilizing factor.

Results of Dunaway and Segal (83) and us (chapter VI) show a possible relationship between the stabilizing factor and GSH. Despite this, it is our present belief that the stabilizing factor probably has little or nothing to do with GSH, but that the latter simply co-purifies with the factor through most of the purification protocol. In addition to our above demonstration that GSH or GSSG cannot replace the factor, this view is supported by a number of other observations.

When the stabilizing factor was titrated with DTNB, we could not find any free sulfhydryl groups (<0.05 per free aminogroup), and none were liberated by prior reduction with DTT. To further explore the nature of a possible sulfur moiety in the stabilizing factor, we treated the factor with iodoacetamide. After treatment a slight excess of cysteine was added to remove excess iodoacetamide. The result (Table X) shows that the stabilizing factor is not sensitive to

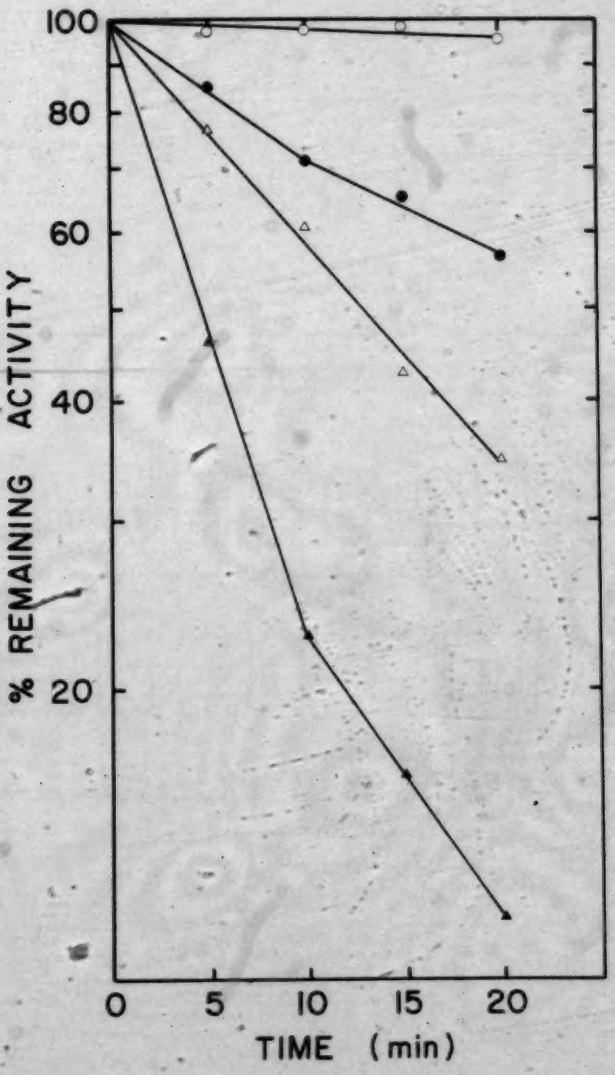

Fig.33. Inactivation of ATP citrate lyase by iodoacetamide. 20 µl enzyme (1 mg/ml) was mixed with 40 µl 20 mM sodium phosphate (pH 8.0). 10 µl iodoacetamide in 0.5 M sodium phosphate (pH 8.0) was added to start the reaction and the mixture was incubated at 25°. Samples were removed at time intervals for assay of ATP citrate lyase. Control (○), 0.1 mM (●), 1.0 mM (△) and 10 mM (▲) iodoacetamide. The rate of inactivation at each concentration of iodoacetamide was determined in triplicate.

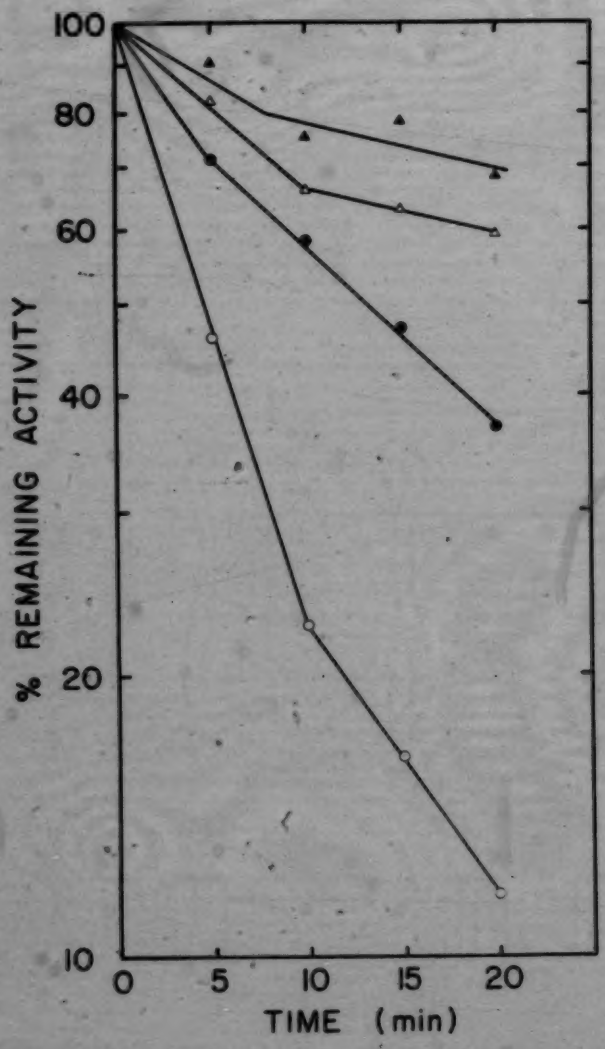

Fig.34. Effect of stabilizing factor on the inactivation of ATP citrate lyase by iodoacetamide. Enzyme was incubated with 10 mM iodoacetamide (○) as described in the legend to Fig.33. The effect of the stabilizing factor was tested by substituting 20 mM sodium phosphate (pH 8.0) with factor in the same buffer. Final concentrations of stabilizing factor are as follows: 0.63 mM (●), 1.3 mM (△) and 2.5 mM (▲). Triplicate experiments were done at each concentration of factor.

iodoacetamide treatment at alkaline pH, indicating the absence of free sulfhydryl groups.

Characterization of preparations of the stabilizing factor (chapter VI) indicates the presence of carbohydrate. It was considered possible that a carbohydrate moiety might be a necessary part of the factor, perhaps linked to the sulfur group of cysteine in GSH in a thioester bond. Hydroxylamine is known to react with esters and produce a hydroxamic acid derivative with the carbonyl function thereby liberating a free alcohol (165). Treatment of the factor with hydroxylamine did not lead to loss of stabilizing activity (Table X). Liberation of free sulfhydryl groups was tested by the method of Uotila (168) in which DTNB is present and the reaction followed at 412 nm. This was unsuccessful, however, since our hydroxylamine solution reacted with the DTNB in the absence of factor and produced 2 moles of thionitrobenzoate for each mole of DTNB.

The stabilizing factor that we have prepared (chapter VI) seems to be different from that of Dunaway and Segal (83). Differences were noted especially in its size and ultraviolet spectrum. Dunaway and Segal (83) found that boiling for 10 minutes destroyed half of the activity and all of the activity was lost upon pronase digestion. They also reported that the activity was lost fairly rapidly without glucose present in the solution. In contrast we have found no loss of activity if the factor is stored without glucose. We have also kept the factor at 37° more than 15

TABLE X

Effect of various treatments on the activity of the stabilizing factor[1]

Treatment	Time of treatment (hours) 0	0.5	1.0	1.5	4.0
Iodoacetamide	62[2]	-	-	-	53
Hydroxylamine	70	-	76	-	56
Heat, 100°					
After Sephadex G-50	53	32	-	-	-
After Sephadex G-15	64	-	63	-	-
Pronase					
After Sephadex G-50	56	52	-	67	-
After Sephadex G-15	43	46	-	51	-

[1] Stabilizing factor, which had undergone treatment for the indicated period of time, was incubated with ATP citrate lyase. Standard assay condition was 20 µl ATP citrate lyase (1 mg/ml) plus 40 µl stabilizing factor in 20 mM sodium phosphate (pH 8.0) plus 10 µl 0.4 M Tris-HCl (pH 8.4) and incubation at 37°. The value shown is the activity of ATP citrate lyase remaining after 30 min. (in %), and is the average of three experiments. The standard deviation ranged from 3.7% to 7.9% Test of the factor was performed in the range of factor concentration, where the standard curve is linear (see Fig.30)

[2] Iodoacetamide was first allowed to react with cysteine before the addition of stabilizing factor and test of the activity at zero time.

hours without any loss of activity. These apparent differences led us to investigate our stabilizing factor in its stability towards heat and pronase. As can be seen (Table X), our stabilizing factor pooled after a Sephadex G-15 filtration run in the absence of glucose can be boiled for an hour without any loss in activity. The same was found for factor prepared in the presence of glucose up to and including the Sephadex G-50 filtration according to the method of Dunaway and Segal (83). Similarly, the activity of none of the preparations was affected by pronase[1].

3. A search for the identity of the stabilizing factor.

A search for other potential stabilizers of the thermal inactivation of ATP citrate lyase was initiated. Since the conditions for thermal inactivation (i.e. 37° and pH 8.3 or above) are the same for maximum activity of the enzyme, we decided to test the substrates for ATP citrate lyase. When the substrates were tested at saturating concentrations, citrate and CoA was found to induce thermal stability of ATP citrate lyase (Table XI). This raised the possibility that these metabolites might be present in the factor solution in significant quantities. The assay for CoA described under

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[1]In the original purification, the homogenized rat liver supernatant is heat treated for 1 minute at 60°. When we extend this to 15 minutes, more than half of the activity is lost. This treatment leads to massive precipitation of proteins and perhaps the stabilizing factor is trapped in this flocculation and coprecipitates with unwanted proteins, accounting for the loss of activity at that point

TABLE XI

Effect of substrates on the thermal inactivation of ATP citrate lyase

Substrates	Final concentration (mM)	Activity remaining after 30 min. (%)[1]
Citrate	3	11.3 ± 4.2
	20	71.8 ± 3.4
CoA	0.01	3.9 ± 0.6
	0.05	40.5 ± 2.2
	0.1	88.3 ± 2.6
ATP	5	11.5 ± 4.3
$MgCl_2$	10	3.8 ± 0.8
ATP + $MgCl_2$	5 + 10	7.2 ± 0.9

[1] 20 µl ATP citrate lyase (1 mg/ml) was incubated with 40 µl of substrate in 20 mM sodium phosphate (pH 8.0) and 10 µl 0.4 M Tris-HCl (pH 8.4) at 37°. Samples were removed at time intervals for assay of ATP citrate lyase. The percent activity remaining after 30 min. was calculated using linear regression analysis on semilogarithmic plot and is the average of triplicate determinations plus standard deviation.

"Methods" is sensitive enough to detect 0.1 nmoles of CoA (Fig.35). When the factor solution was tested for the presence of CoA, none could be detected. If CoA is present there is less than 0.5 µM, an amount having no stabilizing activity on the enzyme. The same factor solution contained 1.63 mM stabilizing factor.

An equivalent method for citrate determination was devised. ATP citrate lyase is known to show a biphasic Lineweaver-Burk plot, when citrate is the varying substrate (124), which becomes linear in the presence of 0.25 M KCl. At high ionic strength we were able to detect as little as 1 nmoles of citrate (Fig.36)[2]. When the factor was tested for the presence of citrate the absorbance change was not different from the blank. Thus it can be concluded that if citrate is present, its concentration is less than 3,5 µM, an amount which does not show any stabilizing activity.

A variety of other compounds have been tested in their ability to stabilize ATP citrate lyase against thermal inactivation (Table XII). Cottam and Srere (131) have shown that when GSSG is added to an assay mixture of rat liver ATP citrate lyase a marked time-dependent inhibition is observed which is reversed by DTT. It is clear that the enzyme is sensitive to disulfide inhibition and consequently it is not surprising that GSSG has no stabilizing effect during the thermal inactivation of ATP citrate lyase (Table XII).

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[2]It seems, however, that the enzyme contained some internal bound citrate, since we observed an absorbance change without the addition of citrate

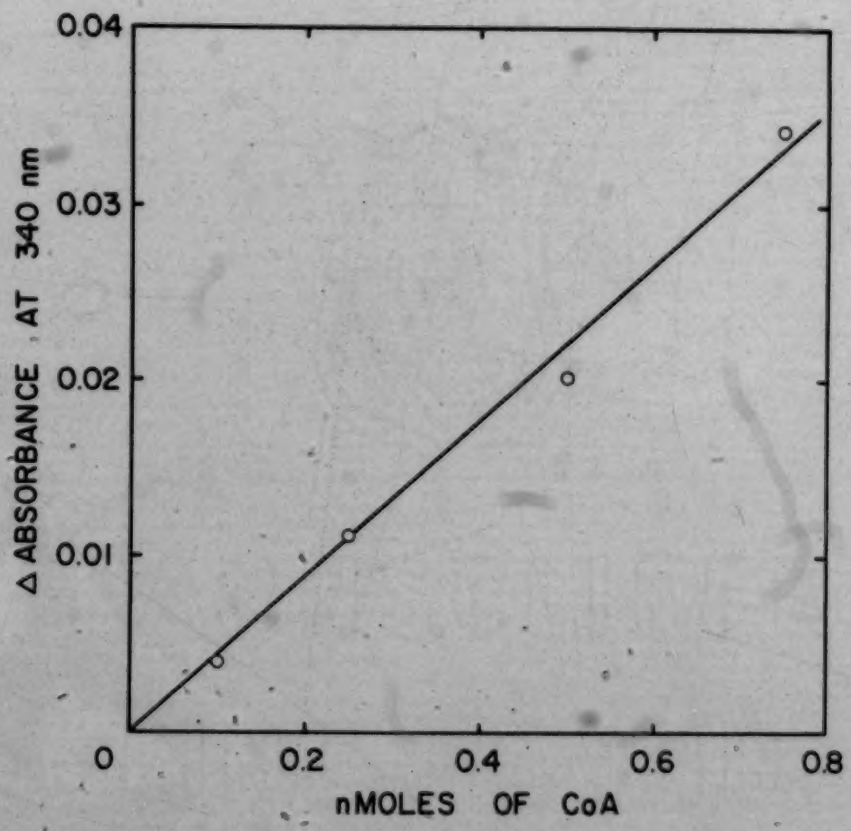

Fig.35. Assay of CoA with ATP citrate lyase. The enzyme was assayed with the amount of CoA varied (for details of assay conditions see under "Method"). The reaction was started by the addition of enzyme and allowed to go until completion. The difference in absorption at 340 nm before and after the reaction was plotted against the amount of CoA added.

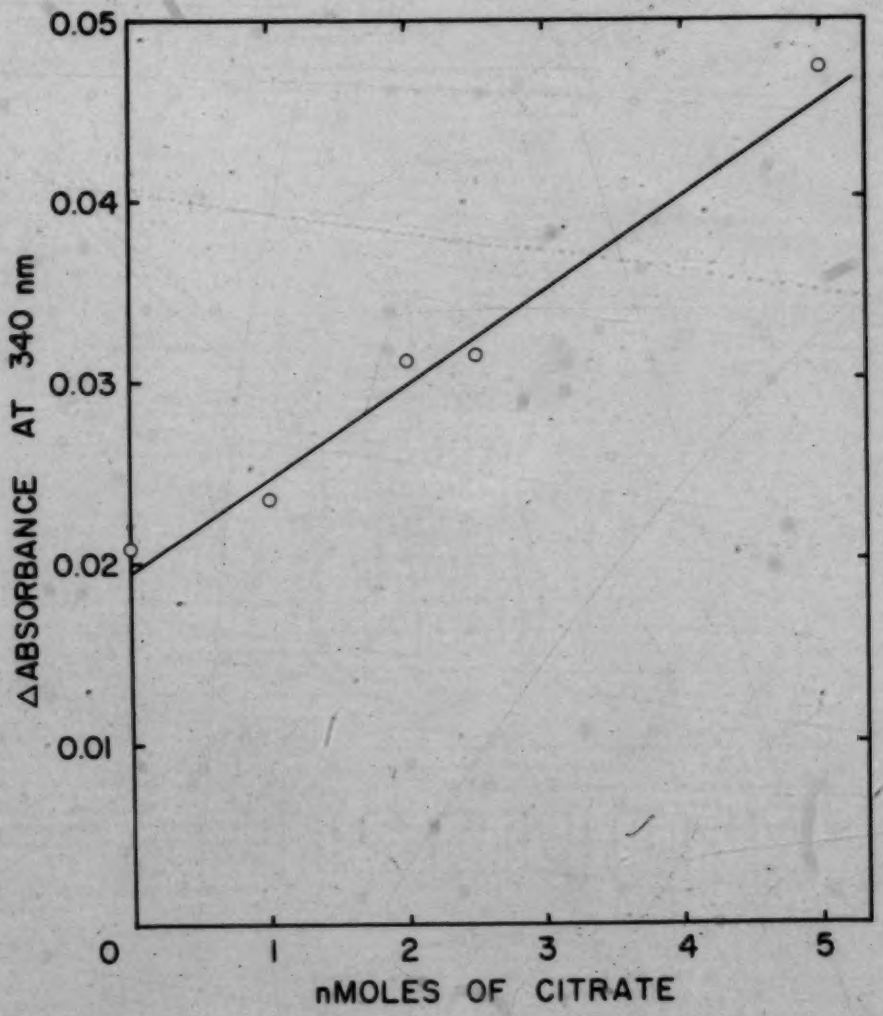

Fig.36. Assay of citrate with ATP citrate lyase. The enzyme was assayed with the amount of citrate varied (for assay conditions see "Method"). The reaction was started by the addition of enzyme and allowed to go until completion. The difference in absorption before and after the reaction was plotted against the amount of citrate added.

TABLE XII

Effect of various substances on the thermal inactivation of ATP citrate lyase[1]

Substance	Final concentration (mM)	Activity remaining after 30 min. (%)
GSSG	1.0 - 8.4	3.1 ± 0.7
GSH + glucuronic acid (1:5 molar ratio)	1.1 : 5.5	19.1 ± 3.2
	2.1 : 10.5	34.0 ± 3.1
	4.2 : 21.0	60.7 ± 4.6
	6.3 : 31.5	71.1 ± 1.4
	8.4 : 42.0	67.5 ± 1.8
GTF	0.1 - 100	3.5 ± 0.6
$CaCl_2$, NH_4Cl, $MgCl_2$, $MnCl_2$, or $FeCl_2$.	2.0	3.6 ± 0.6
Biotin	1.0	3.4 ± 0.3
	5.0	12.0 ± 4.4
Pyridoxal-phosphate	0.1	3.4 ± 0.9
	2.0	3.4 ± 0.4

[1] For experimental details see the legend to Table XI.

The sulfhydryl group of GSH is nucleophilic and, for example, can react with an aldehyde function of a carbohydrate to form a thiohemiacetal. This was considered possible because of the presence of carbohydrate in the preparation and the tentative identification of glucuronic acid based on paper chromatography of the stabilizing factor (chapter VI). GSH was mixed with an excess of glucuronic acid and tested for stabilizing activity, but it was not different from GSH alone (Table XII). The same result was obtained with glucose and galacturonic acid (not shown).

Another possible candidate that was eliminated was the so-called Glucose Tolerance Factor (GTF). This material is composed of glu, gly, cys (1 mole of each) and 2 moles of nicotinic acid complexed to chromium (171). GTF has been reported to be required to maintain normal glucose tolerance and GTF deficiency is characterized by delayed removal of glucose from the blood stream (161,162,171). A chromium complex with GTF activity was prepared according to the procedure of Toepfer *et al* (167) and was tested over a wide range of concentrations for protection of ATP citrate lyase against heat inactivation (Table XII). Under our conditions we could not detect any stabilization.

Several cations were tested as their chloride salts for protection of the enzyme against thermal inactivation (Table XII). Of the metals tested, none showed any protection.

Finally we tested two cofactors, biotin and pyridoxal phosphate. It has been shown that biotin deficiency reduces

the activity of ATP citrate lyase and malic enzyme in avian liver (172). The presence of either of these cofactors did not modify the thermal inactivation of ATP citrate lyase (Table XII).

D. DISCUSSION

Cottam and Srere (131) showed that the loss of most of the activity of ATP citrate lyase that occurs upon reaction with DTNB is correlated to modification of 6-8 of the 50 or so sulfhydryl groups present in the enzyme. It is possible that the enzyme may be oxidized on storage with formation of disulfides that can easily be reduced by a thiol reagent.

The thermal inactivation of ATP citrate lyase is very rapid above pH 8.3. It can be retarded and even reversed by the addition of thiol reagents, especially DTT. While the mechanism in this case probably involves an oxidation-reduction, it is clear that the stabilizing factor works differently. The addition of the factor during the inactivation process apparently halts the process at that state. We suggest that the factor may be able to interact with the remaining sulfhydryl groups inhibiting their further oxidation or participation in inactivation.

Such a protective role of the stabilizing factor is further supported by the observation that it can protect against loss of activity caused by iodoacetamide. Some or all of the sensitive sulfhydryl groups may be located near the active site of the enzyme, since Cottam and Srere (131) showed that the inactivation of ATP citrate lyase by DTNB could be partially prevented by Mg-citrate. That the factor is identical to citrate can be excluded, since citrate in sufficient quantity to stabilize the thermal inactivation of ATP citrate lyase, could not be detected in the factor

solution.

How then is the stabilizing factor able to protect the important sulfhydryl residues from oxidation? The fact that no free sulfhydryl groups can be found on the factor or can be liberated by its reduction argues against mixed disulfide formation between the factor and the enzyme, although such a mechanism could explain the apparent thiol reguirement for the effectiveness of the stabilizing factor. The additive effect of GSH and the stabilizing factor can be viewed as comprised of two processes working simultaneously or sequentially, i.e., reduction by GSH and protection of free sulfhydryl groups by the stabilizing factor.

The identity of the factor is still a puzzle. A variety of compounds has been tested and eliminated as stabilizing candidates. The Glucose Tolerance Factor seemed an exciting prospect, since it is known to have an amino acid composition similar to the stabilizing factor and shows a definite absorption maximum around 260 nm (167). The similarity between the UV-spectrum of the P-component of the factor preparation that was eluted from a paper chromatogram (chapter VI) and that of GTF is very striking. A synthetic chromium complex, which is known to have GTF activity did, however, not show any stabilizing activity. GTF should not be eliminated all together, however, since differences could conceivably exist between the synthetic and the natural product.

In a study of phospoenolpyruvate carboxykinase from rat

liver, Ballard and Hopgood (72) were especially interested in the initial sequence of degradation of this enzyme. *In vitro* inactivation of this enzyme proceeded rapidly with heat, trypsin and a microsomal fraction of rat liver. The inactivation was accelerated by cystine and to a lesser extent by GSSG and retarded by DTT and GSH. Ballard and Hopgood (72) speculated that inactivation by a thiol-disulfide exchange reaction preceded any proteolytic degradation. They also found that inactivation by heat and by the microsomal fraction, but not by trypsin, could be retarded by Mn^{2+} and Co^{2+}, cations that can function as reactants in the enzymatic reaction. Binding of these cations may promote folding of phosphoenolpyruvate carboxykinase into a conformation that is more stable.

Some different cations were tested for their effect on the thermal inactivation of ATP citrate lyase, but no stabilization could be observed. This does not preclude a cation being the actual stabilizing factor, since only a limited number were tested and also a cation might not act independently. Such a case has been proposed by Lardy and coworkers (173), who have isolated an activation protein ("Ferroactivator") of phosphoenolpyruvate carboxykinase which requires Fe^{2+} for activity. In agreement with this concept, Ballard and Hopgood (72) showed no direct effect of Fe^{2+} upon the *in vitro* inactivation of phosphoenolpyruvate carboxykinase.

Dunaway and Segal (83) found their stabilizing factor

to be sensitive to pronase digestion and to high temperatures. In contrast we found that all of our stabilizing activity could be recovered after boiling for 1 hour and after extensive pronase digestion. As controls, the same treatments were performed on factor preparation prepared up to and including the Sephadex G-50 filtration step. This preparation was also stable. Only at the heat treatment step of the original purification, did we lose stabilizing activity for ATP citrate lyase, perhaps simply by physical entrapment in the protein precipitate.

The possibility must then be considered that the stabilizing factor for phosphofructokinase is not the same as the one for ATP citrate lyase. Future work will be required to solve this question.

VIII. CONCLUDING REMARKS

ATP citrate lyase occupies a position in lipogenesis where it could direct the overflow of catabolizable metabolites into fatty acids and triglycerides to be stored and reutilized at times of energy restriction. ATP citrate lyase is not the only enzyme which responds when starved rats are refed a high carbohydrate diet or when diabetic rats are treated with insulin. A whole set of enzymes respond in parallel by an increase in their levels. Insulin, thought to be the primary signal in this induction, does not enter the cell. When insulin reaches the target cell the interaction between the hormone and its receptor brings about a signal to the interior of the cell. The identity of this signal is still a mystery. Many suggestions have been put forward but none of them has held up to close scrutiny (34).

Many different biological processes are influenced by insulin. Some of these responses occur within minutes, such as facilitated glucose transport and activation of certain enzymes, while others take hours and days to exert their influence on the metabolic processes. An example of the latter response is protein turnover. Still obscure is the intermediary link or links between the insulin contact with the cell and the final responses, be they a change in the catalytic activity, a change in the rate of protein turnover, or other responses.

In the time period between the elevation of circulating insulin and the final attainment of high levels of lipogenic

enzymes, the level of the stabilizing factor increases to several times above normal levels. This sequence of events argues for an important function of the stabilizing factor. The proposal that the resultant action of insulin is a retardation of the rate of degradation of specifically lipogenic enzymes during the induction process has been ratified in the case of phosphofructokinase. As a consequence, a topic for future research is the determination of the rate of degradation of ATP citrate lyase under different dietary and hormonal conditions.

In order to understand the role of the stabilizing factor, its origin and its interaction with its target enzymes, it is necessary to know the chemical identity of the factor. While its chemical structure is still not at hand at the conclusion of this work, much information has been obtained. Two facts can be emphasized as a result of the present investigation. 1) The molecular size of the stabilizing factor is much smaller than originally estimated by Dunaway and Segal (83). The discrepancy can probably be related to the presence of glucose in the medium. 2) The stabilizing factor is not glutathione *per se* and is unlikely to be a derivative of glutathione, although this possibility has not been excluded. In light of the present knowledge, we hope that the identification of the stabilizing factor can be achieved in the near future.

In the final days of preparation of this thesis Larner *et al* (178) reported the discovery of a factor from skeletal

muscle generated in the presence of insulin. The factor was named "insulin-mediator" and was shown to have profound inhibitory effect on cyclic-AMP-dependent protein kinase but not on cyclic-AMP-independent kinase. The mediator was also able to stimulate glycogen synthetase phosphoprotein phosphatase. A simultaneous publication by Jarret and Seals (179) revealed that apparently the same mediator was able to stimulate pyruvate dehydrogenase in adipocyte mitochondrial subcellular fraction. The insulin-mediator was found to be heat-stable, a property which was used in the purification. The elution on a Sephadex G-25 column corresponded with a major 230-nm peak and a ninhydrin-positive peak and the molecular weight of the mediator was estimated to be about 1,000 to 1,500 (178). These properties are strikingly similar to the properties of the stabilizing factor.

It is reasonable to argue that intracellular insulin action is mediated by a second messenger. Do the insulin-mediator substance or the stabilizing factor or both function as intracellular messenger for insulin? Are they the same substance?

A discussion of insulin action cannot be complete without mentioning several recent published reports about protein phosphorylation specifically stimulated by insulin. Studies of protein phosphorylation in adipose and liver cells have implied the presence of an insulin-dependent cyclic-AMP-independent protein kinase system (150,169,170). In adipocytes and hepatocytes insulin stimulates selectively

the phosphorylation of a polypeptide of approximately 125,000 molecular weight (109,150). The polypeptide has been isolated and identified as the subunit of ATP citrate lyase (108,109). The insulin stimulation is specific but also adrenalin and glucagon can increase the degree of phosphorylation of the enzyme while at the same time promoting phosphorylation of other proteins. The effect of glucagon has been confirmed by Janski *et al* (110) who have used an immunochemical approach for rapid isolation of ATP citrate lyase from hepatocytes.

The role of structural phosphorylation of ATP citrate lyase is not clear. It occurs on a serine residue and is quite distinct from the phosphorylated catalytic intermediate. The enzymatic activity is not altered by phosphorylation so a metabolic role for this process has to be sought elsewhere. Phosphorylation could cause some structural alteration of the enzyme and thereby influence its susceptibility to proteolytic degradation. Since alteration of enzyme content occurs under conditions where the insulin/glucagon ratio varies and since the level of an enzyme is determined by both its synthesis and its degradation, one can speculate that phosphorylation of existing enzyme molecules may change the rate of degradation of the enzyme. It is of great importance to establish the rate of degradation of ATP citrate lyase during a starvation-refeeding schedule. Furthermore, a test of the *in vitro* stability of phosphoenzyme and dephosphoenzyme can perhaps

clarify the physiological role of the phosphorylation mechanism.

The discoveries of a stabilizing factor, an insulin-mediator and insulin-dependent phosphorylation of ATP citrate lyase are perhaps all linked to intracellular regulatory processes in a coordinated fashion. Further research will undoubtedly help to resolve these questions and lead to a deeper understanding of insulin action in molecular terms.

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